1992

Virucidal activities of amphipathic compounds against lipid-containing bacteriophages

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TITLE:

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DATE: May 31, 1992

VIRUCIDAL ACTIVITIES OF AMPHIPATHIC COMPOUNDS AGAINST LIPID-CONTAINING BACTERIOPHAGES.

by

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A Thesis

Presented to the Graduate Committee

of Lehigh University

in Candidacy for the Degree of

Master of Science

in

Molecular Biology

Lehigh University

April 25, 1992

This thesis is accepted and approved in partial fulfillment of the requirements for the Master of Science

13 May 1992
Date

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Professor Vassie Ware

Acknowledgements

I am deeply greatful to the entire faculty of the Molecular Biology department. Each faculty member, in their own way, contributed greatly to my academic growth. This includes members of my graduate committe Dr. Vassie Ware and Dr. Steven Krawiec. It also includes several professors, Dr. Michael Kuchka, Dr. Jack Abel, and Dr. Bruce Hargreaves who also provided inspiration and intellectual insights in both seminars and coursework. I am thankful to Lehigh University for providing me with the opportunity to obtain the education that I tremendously desired.

I am especially greatful to Dr. Jeffrey Sands. My interest in virology and antiviral research has been strongly motivated by my own personal suffering with viral diseases and the development of a subsequent compassion for human suffering. I am fortunate that Dr. Sands' background as a mentor has included virology and antiviral research, but more importantly that he is sensitive to the need for human compassion behind all science, and that along with his teaching skills he has provided me with an endless amount of support and opportunities.

I have heard it said that science stands on the shoulders of those who came before. Ultimately, within this inverted human pyramid, I hope there is some greater direction. What is science up to without compassion? To reduce error is science but to be motivated by compassion is divine.

I dedicate this humble thesis to all those scientists whose contributions to some cure were motivated by compassion and to all those individuals who have suffered and died before a cure had come. I would like to draw upon their collective voice, which I hear so well, for the enormous amount of support needed to contribute some iota of good, desperately needed, in this world.

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Abbreviations

AV - Anti-Viral
AVC - Anti-Viral-Compound (general)
VA - Virucidal Agent (acts on virus)
Subscripts for AV, AVC, VA:
- cc - critical concentration to begin to see 70.7% virucidal activity
- cm - concentration to achieve 99% or greater virucidal activity
- cp - peak concentrations at which no higher concentrations will yield any greater percent virucidal activity

IC - Incubation Concentration
FC - Final Concentration
Subscripts for IC, FC:
- v - virus (PFU/ml)
- p - a potential but undesirable virucidal agent
- pmax - highest concentration not to exceed where the potential virucidal activities are guarenteed not to occur (M)
- a - anti-viral compound being tested (M)
- i - inert, 100% non-virucidal compounds (M)
- n - virucidal nuetralizing agents (M)
- s - synergistic agent amplifying effects of an anti-viral compound (M)

IR - Incubation Ratio
FR - Final Ratio. Subscripts for IR, FR:
- v/a - virus (PFU/ml) / anti-viral compound (M)
- v/p - virus (PFU/ml) /

IPCO - Incubation to Plated Concentration Overlay
IC that have equivalent FC due to dilution / concentration ranges.
IPCO(n,m) identifies plate n, [n] < [m], whose incubation concentrations (IC) are equivalent to plate m Final Concentrations FC.

IT - Incubation Temperature
It - Incubation time
PC - plaque count
DP - date plated
ET - elapsed time until plaque count
NHL - no host lawn
BP - bad plate
NPM - no plate made
oo - completely plaqued out
>>> - far too many plaques to count @>1000
>> - many plaques > 500

Abstract

The isolation and identification of effective antiviral compounds that do not harm host cell activity is difficult. Small changes in functional groups or molecular conformation of a compound can dramatically affect its virucidal activity and cytotoxicity. Greater complexity is encountered in *in-vivo* biological systems than in *in-vitro* model test systems.

This research has examined the virucidal activity of amphipathic compounds upon the lipid-enveloped phage Φ6 and the protein-coated, lipid-containing phage PM2. Glycyrrhizin and seven selected retinoids, all amphipathic compounds, were investigated to determine their virucidal activity.

In this research, the results of plaque assay experiments of glycyrrhizin on phage Φ6 showed a 98% inactivation at concentrations as low as 1.0μM and up to 100% inactivation at concentrations at or above 10.0μM with no host cell cytotoxicity at concentrations as high as 10mM. Glycyrrhizin showed virucidal effects against the protein-coated phage PM2 but these results were less effective when compared to the inactivation results upon the external lipid-enveloped phage Φ6.

To characterize the effects of *in-vivo* complexities, two factors were examined. Previous work in this lab has shown that temperature and fatty acids could effect the activity of a virucidal compound. In this study, each of these were seperately examined and modified within the *in-vitro* phage Φ6 test system. In this study, incubation temperature was varied and the effect upon the virucidal activity of glycyrrhizin was determined. Unexpectedly, temperature had no appreciable effect on the virucidal activity of glycyrrhizin. Also in this study, individual exogenous lipids were added prior to incubation with glycyrrhizin. Some of the lipids tested; such as arachidylic acid, phosphatidyl choline and cholesterol; are capable of providing a substantial protective effect at ratios as low as 1:100 (lipid:glycyrrhizin) against the virucidal activity of glycyrrhizin. Other exogenous lipids tested; such as stearic acid, oleic acid, and caprylic acid; did not show any interference with the virucidal activity of glycyrrhizin.

Previous work in this lab has shown that the all-trans isomers of retinal, retinol and retinoic acid have virucidal effects. Retinoic acid (all-trans) showed the greatest and substantial virucidal activity against the phage PM2. Retinal and retinol showed the greatest virucidal activity against phage Φ6. Recently a number of cis-isomers of these

same retinoids became available and in this research their virucidal activity was studied and compared to their corresponding all-trans isomer results on the phage PM2. In this research, the 13-cis-isomer of retinoic acid showed an appreciably lower inactivation of the protein-coated phage PM2 compared to the all-trans isomer of retinoic acid. The structural kink in the cis-isomer may reduce the ability of the polar tail to intercalate into the protein coat. In this research, the COOH R group of retinoic acid was the most virucidal retinoid against PM2 among the retinoids tested. Other cis-isomers did not show any increase or overall change in virucidal activity compared to their all-trans isomers against PM2.

The results of this study have added to the appreciation for the complications arising from *in-vivo* system variables, variations in the degree of virucidal activity due to changes in isomeric configurations, and effects of different functional groups against different external viral surfaces. Collectively, these observations can add to the overall understanding needed for the prediction, selection, and design of effective antiviral compounds.

Introduction

1. Background Material

a.) Antiviral Agents

Antiviral agents can act and are therefore classified in several different ways (13, 14, 15). Antiviral or chemotherapeutic compounds can act directly upon a virus particle outside of a host-cell and are termed virucidal agents. Chemotherapeutic compounds that interfere with stages of a viral life cycle such as viral attachment, production, assembly or release are termed virostatic compounds (13). Chemotherapeutic compounds that enhance or aid in the immune system's detection and clearance of a viral presence or infection are called immune adjuvants (17). Subcomponents of a virus particle that solicit a protective level of immune system antibodies are termed vaccines. Current immunization research also includes missense mutations in viral genes, gene incompatabiliy and live attenuated virus technologies (16).

Chemotherapeutic compounds clinically in use today act as virostatic compounds. Current antiviral drugs in clinical use include acyclovir, amantadine and AZT (14, 15). AZT and acyclovir are semi-synthetic nucleotide analogues. While these compounds, at clinical doses, have only mild side- effects they are not 100% effective and do not provide complete clearance of the viral infection (15). More

research is still needed to identify additional antiviral alternatives. While vaccine technology is dramatically advancing, many virus infections do not lend themselves to being prevented by vaccines due to rapid and diverse viral mutations or the inability to target an effective antigenic subcomponent of the virus (16).

It is reasonable to believe that other untested and effective chemotherapeutic agents exist and that the investigation for such compounds is a worthwhile endeavor. There are many sources for chemotherapeutic antiviral agents which include plant sources, animal sources, insect sources, novel microorganism protein and enzyme compounds, organic derivatives or synthetic compounds (14).

Plant sources have long been and still are a major source for numerous classes of pharmaceutical agents (8). Part of the research described in this thesis has focused on a plant source for a novel, specific and identified compound called glycyrrhizin obtained from the Chinese Licorice Plant Root. In addition, the research described here has also examined organic retinoid compounds and several semi-synthetic isomer derivatives.

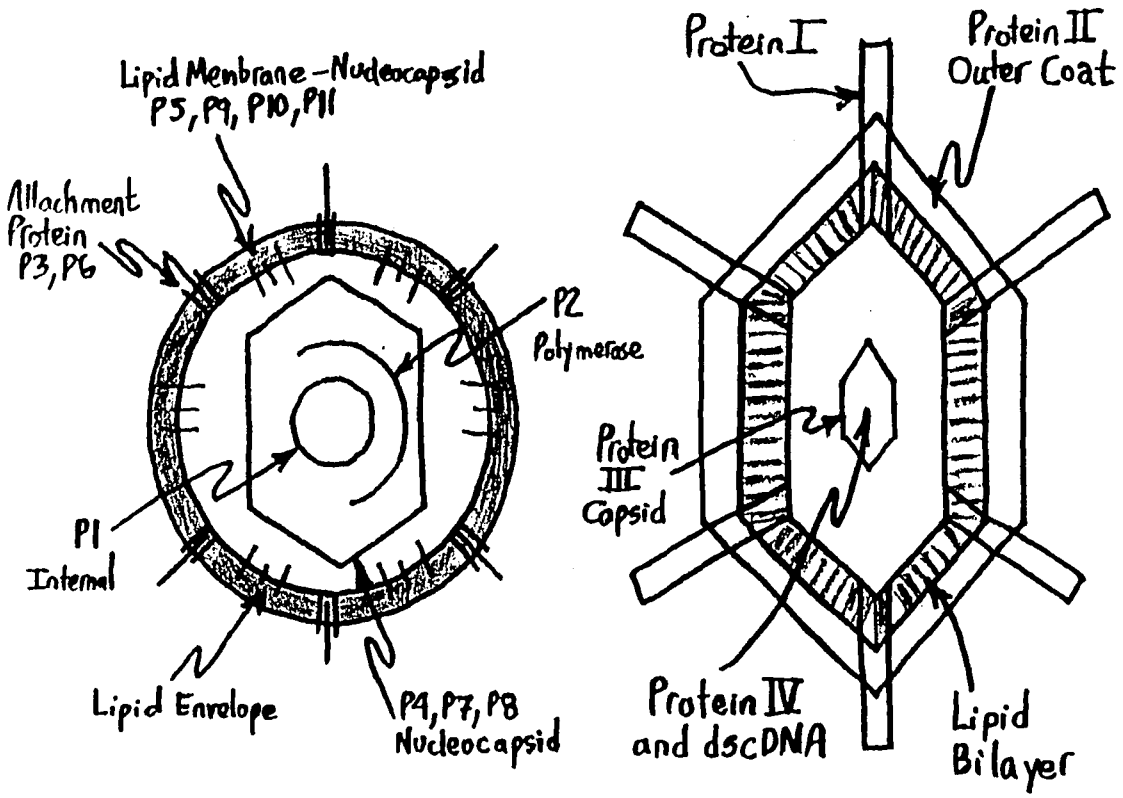
b.) Lipid-Containing Bacteriophages

The discovery and characteristics of lipid-containing bacteriphages began in 1968 with the isolation of PM2 (5) which infects Alteromonas espejiana, a marine bacterium previously termed BAL31 (Pseudomonas) (6). Another lipid-containing bacteriophage, Φ6, which infects Pseudomonas phaseolicola, was discovered in 1973 by Vidaver *et. al.* (7).

The lipid-containing bacteriophages Φ6 and PM2 share some comparative structural characteristics with a few human pathogenic lipid-containing viruses such as herpes simplex virus, influenza, and poxviruses. In addition, PM2 shares some comparative characteristics to protein-coated viruses such as SV40.

The lipid membranes of Φ6 and PM2 are composed of phospholipids that form a bi-layer around the virus to form an envelope. The phospholipids are obtained from the host cell (6). The envelope depicted by hashed region is external in the case of Φ6 and internal for PM2 as depicted in Diagram 1 below. The envelopes have associated proteins which participate as important mechanisms in phage attachment and penetration. The fatty acid composition of each virion is similar to its host cell (2). Both host cells can be grown at room temperature.

Diagram 1: Schematic of Bacteriopahges Φ6 and PM2



Φ6 PM2

The following table summarizes some of the general properties of PM2 and Φ6 bacteriophages depicted in this diagram.

Phage	Genome	Structure	Proteins	Lipid
Φ6	dsRNA M.W. 10E6	Icosahedral core diam 60nm total 75nm	10	External Phosphatidyl- glycerol rich
PM2	dsDNA circular M.W. 6E6	Icosahedral 60nm diam	4	Internal Phosphatidyl- glycerol rich

Bacteriophage PM2 is 60nm in diameter and contains 12% lipid which is found in its internal lipid membrane. The lipid composition of PM2 is 64% phosphatidylglycerol, 27% phosphatidylethanolamine, 7% neutral lipid and 1% acyl-phosphatidylglycerol. The lipid is obtained from the host bacteria Alteromonas espejiana (previously identified as BAL31). BAL31 is a gram-negative marine bacterium and its lipid composition is 75% PE and 23% PG. Phage PM2 lipid composition ratio is not a reflection of the host. Phage PM2's fatty acid composition is similar to its BAL31 host. Phage PM2 is 56.6% $C_{16:1}$, 12% $C_{16:0}$, 13.9% $C_{18:1}$ and 7.3% $C_{17:cy}$ a cysteine sulfoxyl linked fatty acid. 62% of phage PM2 volume is H_2O and 42% of the H_2O is contained in the viral core.

Phage PM2 contains circular double stranded DNA of a molecular weight of 6×10^6. The virion contains four proteins. Two of the virion proteins, I and II, are external. Protein I surrounds the virion lipid membrane and protein II protrudes and extends outside the protein coat of protein I. Two proteins, III and IV, are internal to the lipid membrane in the virion core.

Inactivation experiments using the retinoid, retinoic acid, on PM2 have demonstrated that the acid COOH (COO-) group at the end of the polar tail is the most active

functional group. It is postulated that this functional group interacts with the protein coat and possibly down into the internal lipid bilayer causing virion particle disruption (1, 4).

The adsorption rate of phage PM2 is 3.4×10^{-9} m/min. Synthesis of host DNA stops early in infection but synthesis of viral mRNA stably continues throughout infection. The latent period is about 40-45 minutes. Mature virion particles emerge 36 minutes after infection.

Bacteriophage Φ6 host cell is a prototrophic phytopathogen or a bean plant pathogen, Pseudomonas phaseolicola, previously identified as HB10Y. Phage Φ6 attaches to host pili. The exact structure of all components of Φ6 are not well characterized. Bacteriophage Φ6 contains 3 segments of double stranded RNA of 2.3×10^6 MW, 3.1×10^6 MW, and 5.0×10^6 M.W.. The exterior lipid bilayer structure of the virion is amorphous containing lipid, fatty acid and protein and is 60-70 nm in diameter. The core capsid is icosohedral an measures about 50nm in diameter. The inner DNA containing region comprises a volume diameter of about 30nm and composes 12% of the virion.

Bacteriophage Φ6 is about 25% lipid. Of the 25% lipid

composition of Φ6, 57% is PG, 35% is PE and 8% is cardiolipin (diPG) and acylPG. In the host cell, unlike Φ6, the PE is present in quantities twice that of PG. The fatty acid composition of phage Φ6 is similar to the host. About 61% of the fatty acid composition of Φ6 are saturated composed of $C_{16:1}$ and $C_{18:1}$. About 33% of the fatty acid composition of Φ6 are unsaturated composed of $C_{16:0}$.

Bacteriophage Φ6 codes for 11 known proteins. Proteins P1 and P2 are involved in RNA polymerases activities and constitute 15.6% and 6% of the total protein composition. Protein P3 is the lipid envelope surface attachment protein and comprises 9.8% of the total protein composition. Protein P6 (2%) is also part of the surface attachment protein on the lipid envelope. Protein P8, 26.3%, is used in the structure of the nucleocapsid. Proteins P5 (3%), P9 (18%), P10 (9.5%) and P11 (6%) are part of the amorphous lipid bilayer and interact with the nucleocapsid. Protein P12 is a non-structural protein used in assembly of the envelope. (2,7).

Inactivation of phage Φ6 has been most effective with the retinoids, retinal and retinol. The functional R group at the end of the polar tail interacts with the lipid bilayer causing the release of the attachment protein P3

from the virion envelope without major particle disruption (1, 4).

c.) Glycyrrhizin (a triterpene)

Pharmaceutical compounds have long been isolated from plant sources and are known as phytopharmaceuticals. A number of unexplored plants and the novel compounds they contain may hold promise for treatment or cure of some viral diseases.

About 80% of modern medicines have evolved from herbal or plant medicines (9). Some modern medicines like aspirin, salicylic acid, continue to be the same active component of the original plant extract. The plant, glycyrrhiza glabra; leguminosae, has been used in herbal medicine for 2000 years. The plant root contains novel triterpene molecules in glycoside compounds known as glycyrrhetic acid along with several other related aglycones (10, 11).

Glycyrrhizin or glycyrrhetic acid is a known anti-herpetic compound (8, 12). The compound was shown to be both virucidal and also to interfere with virus replication in cell culture. In addition to HSV-1; VSV, vaccinia, and Newcastle disease virus were all substantially inhibited by glycyrrhizin. These four membrane-containing viruses were inhibited 2 to 3 $\log_{10}$pfu in Hep-2 cells by 8mM glycyrrhizin both immediatly after infection and almost as well at 3 hpi. At this concentration there was no cell cytotoxicity (12).

Glycyrrhizin has been tested, as described in this thesis research, chiefly on the bacteriophage Φ6 for any virucidal activity. The outer lipid membrane structure of Φ6 is grossly similar to that of HSV.

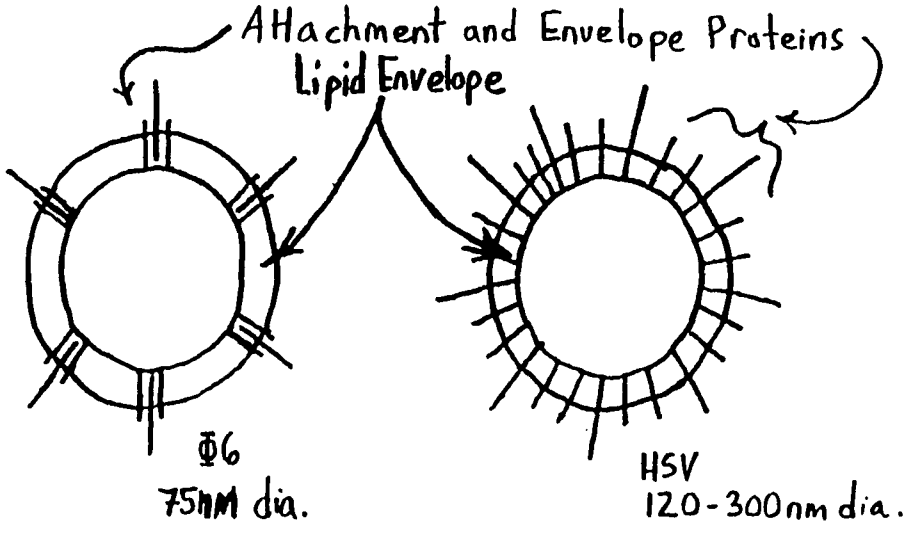
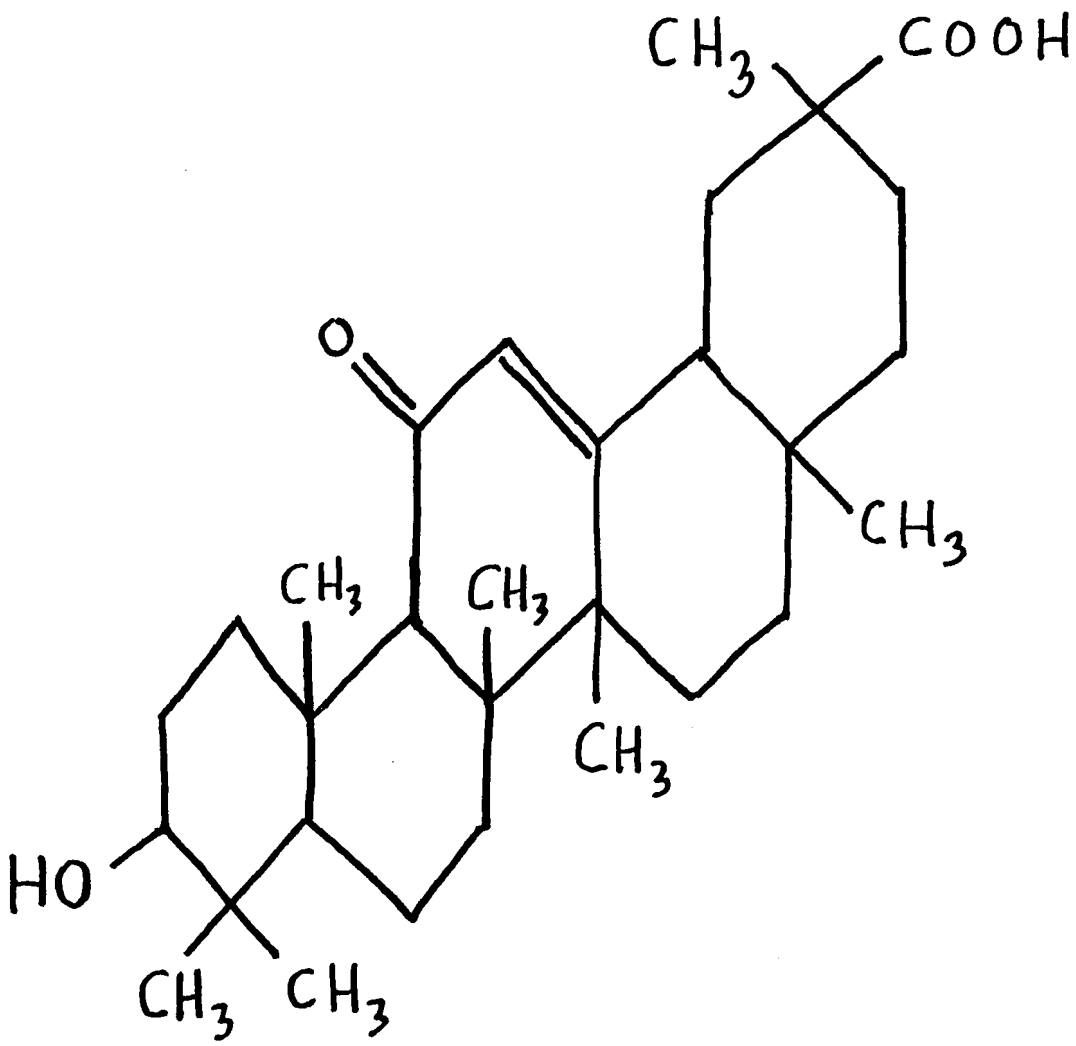


Diagram 2

Comparison of Φ6 and HSV outer envelope structure

The results that have been obtained on the virucidal activity of glycyrrhizin on Φ6 from this research can be compared with the reported results on herpes virus. The interest in testing a potential antiviral compound is not only to determine if antiviral properties exist but also at what concentrations they occur. Additional considerations include whether or not the compound is host-cell cytotoxic at effective antiviral concentrations and what additional factors increase or decrease the antiviral activity of a compound.

Diagram 3. Structure of Glycyrrhetic Acid (Glycyrrhizin)



d.) Retinoids

A number of trans-isomer retinoid compounds were previously tested in earlier experimental work to determine their virucidal activities on both PM2 and Φ6 phages (1, 4). Synthetic retinoids in the cis-isomer conformation were not available at that time.

Retinoic acid was shown to be the most active of the retinoid compounds tested on PM2 with inactivation occuring at 6μM. PM2, whose lipid bilayer is internal surrounded by a protein coat, also showed inactivation by retinal and retinol at concentrations above 175μM but was much less susceptable to the retinol and retinal compounds than Φ6. The mechanisms for inactivation of PM2 are not exactly known but are believed to be caused by particle disruption.

Retinol and retinal (all trans) have been shown to be potent inactivators of Φ6 to greater than 99% inactivation at 0.35μM concentrations within 30 minutes. Phage Φ6 showed a degree of inactivation by retinoic acid but was less susceptable than PM2. The mechanism of inactivation for Φ6 has been shown to be facilitated by the release of an external envelope protein P3 (3, 4). P3 is an attachment protein required for infection. The release of P3 does not cause substantial virus particle disruption (3,4).

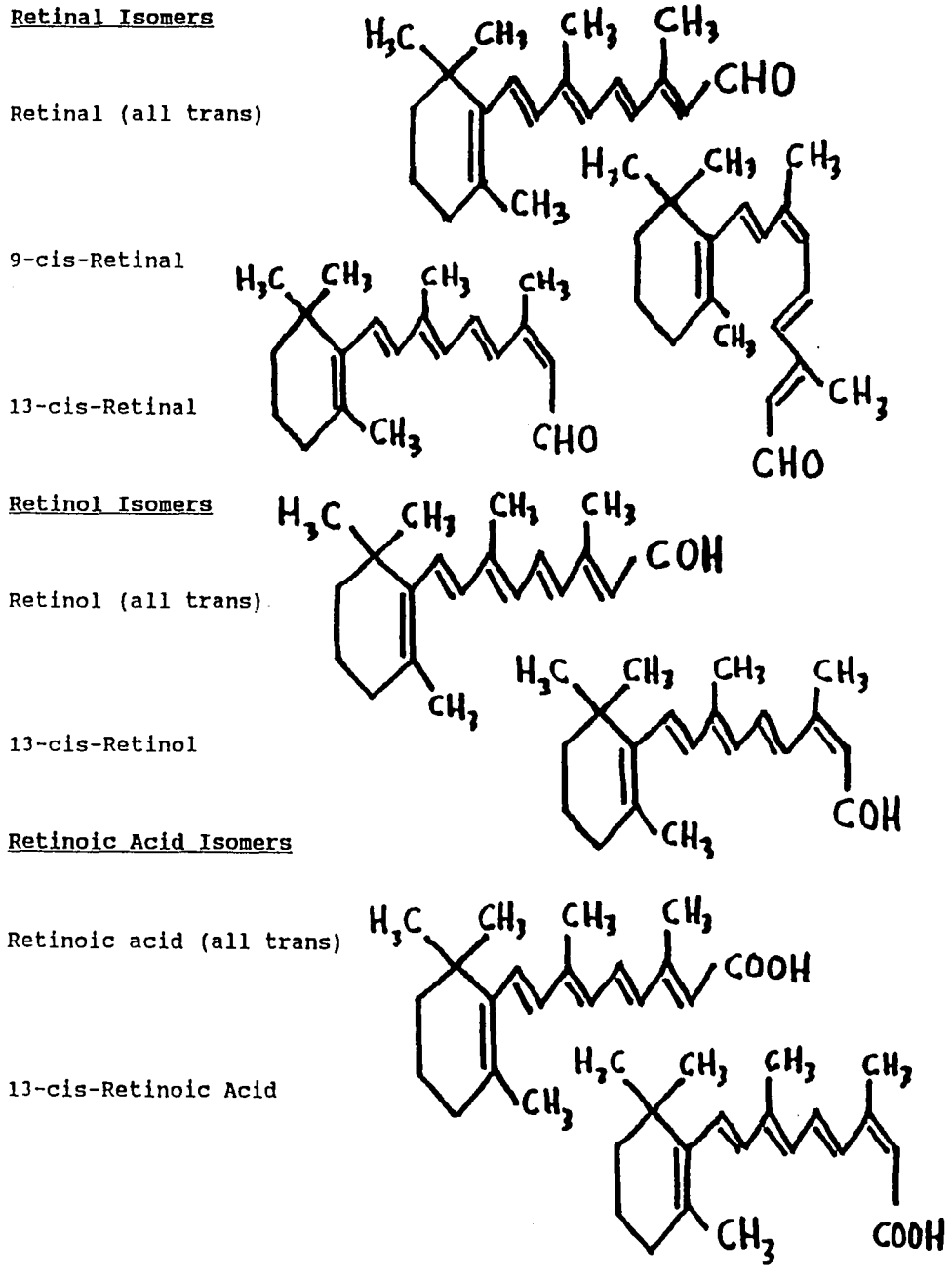
Previous work in this lab had determined the percent inactivation curves of several compounds on phages Φ6 and PM2 and also on the human virus HSV-2 (1). Those results are summarized here. Fifty percent inactivation was achieved by retinol upon Φ6 at a concentration of 0.1μM. Retinol (all-trans), retinal (all-trans), and retinyl acetate achieved 100% inactivation of phage Φ6 by 0.4μM concentrations of each. Retinoic acid (all-trans) achieved 50% inactivation of Φ6 at a concentration of 0.8μM and 100% inactivation at 11.0μM concentration. To achieve 50% inactivation of PM2, greater than 175μM concentrations of retinal (all-trans), retinol (all-trans), retinyl acetate were required. To acheive 50% inactivation of phage PM2 by retinoic acid a concentration of 6μM was required. To achieve 50% inactivation of HSV-2; 0.45μM concentration for retinal (all-trans), 10.0μM concentration for retinol (all-trans), and 33μM concentration for retinoic acid were required. Inactivation experiments upon phage Φ6, phage PM2 and human herpes virus HSV-2 were performed by incubating 5 x 10^5 PFU/ml of phage at 20°C, for 30 minutes and with each respective retinoid concentration.

Several all-trans isomers of the retinoids have been shown to have virucidal effects on PM2, especially retinoic acid. In recent years four cis-isomers of these retinoids have become available. One objective of this research is to

determine the virucidal activity of the four cis-isomers upon phage PM2 and compare the virucidal effectiveness of the cis-isomers to the all-trans isomers.

Shown below are the compounds that were tested for virucidal activity on the PM2 phage in the present study.

Diagram 4. Retinoid Structures



2. Overall Rationale and Research Aim

It is important to determine if a compound shows antiviral activity but more to determine importantly at what concentration does the antiviral activity occur. In addition, it is important to determine and identify what other factors contribute toward or interfere with the antiviral activity of a compound. This research has examined single, controlled factors which affect the virucidal activity of a compound and what kind of generalizations can be proposed for predicting and selecting antiviral agents. In part of this research, two simple variables were individually introduced and their effects on antiviral activity were observed. Both temperature range and the presence of exogenous lipid compounds were individually introduced into an antiviral testing system. Experiments to observe the effects of temperature and exogenous lipids on the virucidal activity of glycyrrhizin were performed. In addition this research has examined two kinds of external viral surfaces and their susceptibleness to amphipathic compounds. A new amphipathic compound was tested on a lipid enveloped phage. Synthetic isomers of retinoids were examined and their virucidal results were compared to their all-trans isomers in order to further elucidate mechanisms of amphipathic retinoid compound inactivation of a protein-coated phage.

Experimental data were collected using the plaque-assay method and the following subsequently determined:

* the degree of virucidal activity of a known anti-herpetic compound glycyrrhizin on Φ6 compared to PM2

* the effects of temperature on the virucidal activities of the antiviral compound glycyrrhizin

* the ability of select exogenous lipids to act as a neutralizing agent against the virucidal activities of the virucidal compound glycyrrhizin

* the degree or lack of recovery of the virucidal activity of glycyrrhizin in the presence of protective levels of a lipid neutralizing agent

* how the cis retinoid isomers compare with the all-trans isomer's virucidal activity on the phage PM2

* which retinoid functional groups show greater virucidal activity on protein-coated phages compared to those functional groups more active on lipid-enveloped phages; including comparisons of their cis isomers

III. Glycyrrhizin and Φ6 Studies

1. Glycyrrhizin antiviral activity upon Φ6

a.) Rationale and Hypothesis

Since glycyrrhizin has been reported effective as a virucidal agent against HSV and both Φ6 and HSV are lipid envelope viruses, I believe that glycyrrhizin is acting on some lipid and protein components on the lipid-envelope of HSV. Phage Φ6 has an attachment protein P3 within its lipid membrane. Lipid envelopes, while composed mostly of lipid, are usually amorphous and contain varying amounts of protein components. Virucidal compounds inactivating Φ6, such as retinol and retinal did so by causing release of with the P3 protein from the lipid membrane (1, 4). I had hypothesized that glycyrrhizin would also be an effective virucidal agent against phage Φ6. The inactivation of Φ6 may suggest that glycyrrhizin might be interacting with lipid and protein within the viral envelope of Φ6 and perhaps other lipid envelope viruses such as HSV. Glycyrrhizin is, like other virucidal compounds, believed to be dislodging the P3 attachment protein. I had also hypothesized that the virucidal activity of glycyrrhizin would be more vigorous upon an external lipid membrane rather than an external protein coat. In this research glycyrrhizin has also been tested on PM2, a protein coated phage. I had hypothesized that glycyrrhizin would be less effective on PM2 than Φ6, due to the lack of an **external** lipid envelope on PM2.

b.) Experimental Design and Method

The general plaque assay method was followed. Tubes containing 5×10^5 PFU/ml of phage Φ6 were prepared at a volume of 10ml. Respective concentrations of glycyrrhizin, listed below, were added at time t=0 and vortexed. All tubes incubated for 30 minutes. Final plating concentrations of 5×10^3 PFU/ml and 5×10^1 PFU/ml of the above tubes were made by dilution. A volume of 0.1ml of phage concentration samples were added to 10ml top agar tubes which already contained 0.4ml of bacterial host which was then vortexed and immediatly plated. Top agar tubes were cooled to below 45°C before either host or phage were added. Immediatly after host and phage were added to the cooling top agar, the incubation tubes were vortexed and then plated. Plates sat for 24-36 hours and then plaques were counted.

Several concentrations of glycyrrhizin were used, namely, 0.0μM, 0.1μM, 1.0μM, 10.0μM, 100.0μM. These concentrations were all soluble in V-Medium (see Appendix A) which was used for host cell growth. The 0.0μM concentration of glycyrrhizin acted as a control and to normalize percent inactivation. This yielded an inactivation curve for glycyrrhizin on Φ6.

In addition, experiments were done to test the effects of glycyrrhizin on normal host lawn growth without any phage

present. This was performed to ensure that plaque counts and host lawn morphology is soley due to phage inactivation and not from interference of the glycyrrhizin with normal host growth.

The exact same experimental method was also performed using phage PM2, its respective host and medium, in place of phage Φ6.

c.) Data Section

TABLE 1. Plaque Count Data obtained from Inactivation Studies of Glycyrrhizin upon the bacteriophage Φ6.

PLAQUE COUNT PER PLATE									
Incubation Concentration of Glycyrrhizin	Plated Concentration of Phage Φ6 5×10^3 PFU/ml				5×10^1 PFU/ml				Average Percent Inactivation
	Experimental Runs								
	#1	#2	#3	#4	#1	#2	#3	#4	
0.0 μM	>>>	>>>	>>>	>>>	69	57	90	61	0%
0.1 μM	720	620	>>	>>	9	7	9	6	88%
1.0 μM	480	310	440	471	4	3	4	3	98.5%
10.0 μM	3	1	3	1	0	0	0	0	99.9%
100.0 μM	0	0	0	0	0	0	0	0	100%

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage Φ6 incubation concentration of 5×10^5 PFU/ml.

There were two plated concentrations of phage Φ6 namely, 5×10^3 PFU/ml and 5×10^1 PFU/ml with host HB10Y. The experiment was run four times at five concentrations of glycyrrhizin. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, across all four runs, and a percentage was then based on 0.0μM control at 0% inactivation.

Qualitative Effect 1

Test of Glycyrrhizin on Normal Φ6 Host (HB10Y) Lawn Growth

A concentration of 100μM of glycyrrhizin was added to the top agar of several plates along with host bacteria HB10Y without phage. At the same time, control plates were also prepared with only host bacteria HB10Y and no glycyrrhizin or phage. A comparison of results showed no effect, adverse or otherwise, in normal HB10Y bacterial lawn growth, thus demonstrating that the antiviral activity of glycyrrhizin is not interfering with normal host growth of HB10Y at concentrations well above those that occur after incubation dilutions. Plaque morphology or plaque reduction can be grossly attributed to viral inactivation during incubation.

Comparative Inactivation Study on Phage PM2

TABLE 2. Plaque Count Data obtained from Inactivation Studies of Glycyrrhizin upon the bacteriophage PM2.

PLAQUE COUNT PER PLATE					
Incubation Concentration of Glycyrrhizin	Plated Concentration of Phage PM2 $5x10^3$ PFU/ml		$5x10^1$ PFU/ml		Average Percent Inactivation
	Experimental Runs #1	#2	#1	#2	
0.0 μM	>>>	>>>	58	62	0%
0.1 μM	>>>	>>>	77	66	-19%
1.0 μM	>>>	>>>	65	59	-3%
10.0 μM	>>	>>	41	51	23.3%
100.0 μM	410	480	40	49	25.0%

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of $5x10^5$ PFU/ml.

There were two plated concentrations of phage PM2 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host BAL31. The experiment was run two times at five concentrations of glycyrrhizin. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

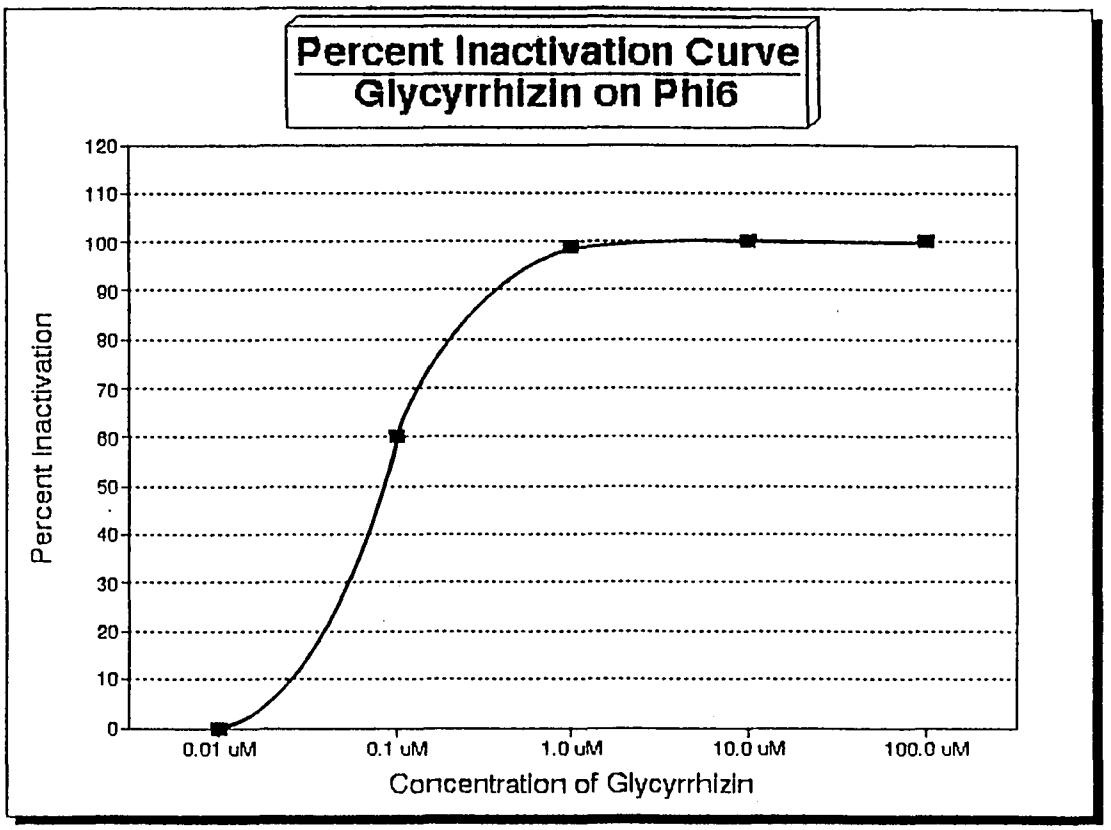
Qualitative Effect 2

Test of Glycyrrhizin on Normal PM2 Host (BAL31) Lawn Growth

A concentration of 100μM of glycyrrhizin was added to the top agar of several plates along with host bacteria BAL31 without phage. At the same time, control plates were also prepared with only host bacteria BAL31 and no glycyrrhizin or phage. A comparison of results showed no effect, adverse or otherwise, in normal (BAL31) bacterial lawn growth, thus demonstrating that the antiviral activity of glycyrrhizin is not interfering with normal host growth of BAL31 at concentrations well above those that occur after incubation dilutions. Plaque morphology or plaque reduction can be grossly attributed to viral inactivation during incubation.

d.) Graph Section

GRAPH 1. Percent Inactivation Curve for Glycyrrhizin on Φ6.



The above inactivation curve was obtained from data using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml and at five concentrations of the compound glycyrrhizin.

There were two plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host HB10Y. The experiment was run four times at five concentrations of glycyrrhizin. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, across all four runs, and a percentage was then based on the 0.0μM control value of 0% inactivation. A percent inactivation was determined for all five concentrations of glycyrrhizin.

e.) Results Summary

The results indicate a very effective virucidal activity of glycyrrhizin upon phage Φ6. Virucidal activity begins at concentrations as low as 0.1μM of glycyrrhizin yielding a 88% inactivation. A 98.5% inactivation of Φ6 was seen at 1.0μM glycyrrhizin. There is further inactivation, up to 100%, at higher concentrations but for all practical purposes complete inactivation of Φ6 occurs at a 10.0μM concentration of glycyrrhizin with an actual inactivation of 99.9%. The result of the test involving the presence of only glycyrrhizin (no phage) on normal growth of Φ6 host (HB10Y) lawn growth showed no effect, adverse or otherwise.

TABLE 3. Percent Inactivation by Glycyrrhizin upon Φ6 compared to Percent Inactivation upon PM2.

PERCENT INACTIVATION COMPARISON		
Incubation Concentration of Glycyrrhizin	Percent Inactivation	
	Phage PM2	Phage Φ6
0.0 μM	0.0%	0.0%
0.1 μM	- 19.0%	88.0%
1.0 μM	- 3.0%	98.5%
10.0 μM	23.3%	99.9%
100.0 μM	25.0%	100.0%

Table 3 shows the percent inactivation for each phage namely, PM2 and Φ6, at each respective concentration of glycyrrhizin. These data were taken from Table 2 and Table 3.

Results of glycyrrhizin virucidal activity on PM2 show that at concentrations of 0.1μM and 1.0μM there is an anomalous increase in plaque formation. At concentrations of 10μM and 100μM of glycyrrhizin a 24% inactivation of phage PM2 occurs. The percent inactivation of PM2 at respective concentrations of glycyrrhizin is comparatively less than for those of phage Φ6 inactivation results.

f.) Conclusions and Discussion

The objective of this part of the research was to test the virucidal activity of a reported phytopharmaceutical antiviral agent, glycyrrhizin, on two different phage-host systems. Two phage types, comparatively different in structure, where examined. The hypothesis was that the lipid-envelope phage Φ6 would be susceptible to the virucidal activity of glycyrrhizin. In addition the hypothesis included that the lipid enveloped phage Φ6 would be more susceptible to the virucidal activity of glycyrrhizin than the protein-coated phage PM2 would be. In addition there was an important interest in examining just how potent of a virucidal agent glycyrrhizin was on each phage (Φ6 & PM2). Virucidal results from glycyrrhizin on each phage PM2 and Φ6 were to be compared and correlated to:

- the reported work of glycyrrhizin against HSV and
- the similar work of retinoids against Φ6 and PM2.

Finally, as with any virucidal agent, it was essential to verify that the antiviral activity being observed was virucidal and not cytotoxic to the host. Control experiments were performed and did show that glycyrrhizin did not interfere with normal growth of either host.

As seen from the results in Table 1, glycyrrhizin is a potent virucidal agent against Φ6 phage. The Φ6 envelope structure is composed of mostly lipids and some protein receptors as previously described in the introduction. The activity of glycyrrhizin is virucidal and in this research does act upon both phages tested, Φ6 and PM2, and does not interfere with normal growth of either host. As seen from Data Table 2, glycyrrhizin is not as potent of a virucidal agent against the protein-coat phage, PM2; at least when compared to the inactivation results against the lipid-enveloped Φ6. Glycyrrhizin is virucidal to PM2, but at higher concentrations. Most other potent virucidal agents acting on phage Φ6, such as retinal and retinol, (1) have been consistently shown to act by dislodging a susceptible envelope protein. I also believe glycyrrhizin may be acting upon this envelope protein. A more important observation is that the overall exterior structure of the virus may need to be comprised mostly of a lipid composition, such as with Φ6 and HSV. If such is the case, it is an important factor in the intelligent design or selection of virucidal compounds. This is further substantiated because the protein outer-structure of PM2 is not as susceptible to virucidal activity of the amphipathic compounds glycyrrhizin and retinal.

When comparing glycyrrhizin and retinal, in addition to their amphipathic structure, the CHO functional group is the most active functional group, in each of their respective polar regions. Perhaps a consistent requirement for this active functional component of these virucidal molecules is an interaction with a lipid membrane that is external and mostly lipid which containing at least some susceptible functional proteins.

One objective was to determine the inactivation curve for the virucidal activiy of glycyrrhizin upon Φ6. Another objective was to look for a correlation between inactivation results of HSV compared to inactivation results of Φ6 when glycyrrhizin and retinoids were each tested upon both viruses. Glycyrrhizin has been shown (8) to have appreciable antiviral activity (50% phage reduction) against herpes simplex virus (HSV) at concentrations of about 200μM. The positive correlation between the virucidal effects of glycyrrhizin upon both lipid-enveloped viruses Φ6, HSV and a few other lipid-containing virus further substantiates the use of phage Φ6 as an early experimental system for testing potential virucidal agents targeted against lipid-enveloped viruses. The following table takes results from this research and results from both previous and reported data and makes a comparison between retinoid activity upon phage Φ6 and HSV; and glycyrrhizin activity upon Φ6 and HSV.

Table 4. Comparison between Required Concentrations of Glycyrrhizin and several Retinoids upon both the human virus HSV and the bacteriophage Φ6 at which 50% Inactivation of Virus is Achieved.

COMPARISON OF THE VIRUCIDAL CONCENTRATION REQUIRED FOR 50% INACTIVATION OF VIRUS

Virus	Virucidal Compounds			
	Glycyrrhizin	Retinal	Retinol	Retinoic Acid
HSV	200μM	0.45μM	10.0μM	33μM
Φ6	1μM	0.6μM	0.2μM	6.0μM

Table 4 displays results from this research and results from both previous and reported data and makes a comparison between retinoid activity upon Φ6 and HSV; and glycyrrhizin activity upon Φ6 and HSV. The comparison indicates the concentration of each compound required to achieve 50% inactivation of virus.

The collective comparison of the above results does suggest a positive correlation between the virucidal effects of glycyrrhizin and retinoids upon both lipid-enveloped phage Φ6 and the human virus HSV and does further substantiate the use of phage Φ6 as an early experimental system for testing potential virucidal agents targeted against lipid-enveloped viruses.

2. Temperature effects upon The Virucidal Activity of Glycyrrhizin upon Φ6

a.) Rationale and Hypothesis

In an *in-vivo* system the activity of a potential antiviral compound becomes more complicated. In an attempt to be able to measure and control individual and independent variables and yet only add one degree of complexity, the plaque assay method was utilized with a known virucidal compound, glycyrrhizin, upon the phage Φ6 with a temperature variable during incubation with the phage and various concentrations of glycyrrhizin.

Changes in temperature affect both the phage and the antiviral compound during incubation. The objective of this part of the research was to see what and how much of an effect changes in incubation temperature would have on the virucidal activity of glycyrrhizin against phage Φ6. The activities of molecules and the structure of viral membranes may alter the virucidal potential of an antiviral compound. These effects of temperature could be due to structural changes in molecules or simply by changes in physical interactions involved between the viral lipid membrane and glycyrrhizin.

I had hypothesized that in addition to viral lipid membrane reactions to temperature that larger or more complex antiviral molecules, such as glycyrrhizin, would have shown some appreciable degree of change in their virucidal effectiveness due to variations in incubation temperature.

Initial inactivation experiments were performed at room temperature. In this part of this research ranges in incubation temperature above and below room temperature were also investigated. I had hypothesized that at lower temperatures glycyrrhizin would be less virucidal and that at higher incubation temperatures glycyrrhizin would be more virucidal. The reasoning behind this hypothesis is that the Φ6 membrane would be more susceptible to the virucidal activity of glycyrrhizin at higher temperatures because the lipid membrane would be more fluid and therefore allow greater potential for interaction between protein components and glycyrrhizin.

b.) Experimental Design and Method

The general plaque assay method was used at incubation temperatures of 2°C, 5°C, 10°C, 20°C, 30°C, 40°C and 50°C. Percent inactivation was obtained from normalization of PFU/ml plaque assay data obtained from control experiment data.

The phage incubation concentration of 5 x 10^5 PFU/ml was prepared and respective concentrations of 0.0μM, 0.1μM, 1.0μM, 10.0μM and 100.0μM of glycyrrhizin were added. Immediatly after adding glycyrrhizin, at time t=0, the incubation tubes; containing medium, phage and glycyrrhizin; were placed in controlled temperature baths for 30 minutes. Immediately after the 30 minutes, phage dilutions were made from the incubation concentration of 5 x 10^5 PFU/ml to the plating concentrations of 5 x 10^3 PFU/ml and 5 x 10^1 PFU/ml. Appropriate volumes of host cells were added to plating concentrations of phage, vortexed and then immediatly plated. The control for each temperature run experiment contained 5 x 10^5 PFU/ml of phage but no (0.0μM) glycyrrhizin.

A 'Control Experiment' was performed using the plaque assay at an incubation time of 30 minutes, an incubation

temperature at each respective temperature indicated above, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml with no glycyrrhizin present. To eliminate a variable, the 10ml volume of phage used for incubation, at a concentration of $5x10^5$ PFU/ml was, for each incubation temperature, taken from a single dilution stock prepared at a concentration of $5x10^5$ PFU/ml and at a volume of 200ml. The 10ml of phage was taken from the 200ml stock for each individual incubation temperature and all individual incubations at their respective temperatures were run simultaneously. The same stock was used for both runs of the experiment. A percent inactivation was calculated from data obtained from each incubation temperature. The effects of incubation temperature on viral infectivity was obtained. These results were then used to normalize data obtained from independent temperature experiments and to factor out those effects of temperature upon phage alone. These steps allow the derivation of what portion of the changes of virucidal activity were due soley to temperature effects upon glycyrrhizin.

There were two plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host HB10Y. Two experimental runs were made at each plating concentration. A count of plaques per plate was made.

c.) Data Section

TABLE 5-A. Incubation Temperature of 2°C.
Plaque counts per plate for phage Φ6 incubated with glycyrrhizin at 2°C.

PLAQUE COUNT PER PLATE - INCUBATION TEMPERATURE = 2°C

Incubation Concentration of Glycyrrhizin	Plated Concentration of Phage Φ6 $5x10^3$ PFU/ml		$5x10^1$ PFU/ml		Average Normalized to N x 10^3 PFU/ml
	Plates #1	#2	#1	#2	
0.0 μM	20	30	0	0	25
0.1 μM	0	0	0	BP	0
1.0 μM	1	0	0	0	0
10.0 μM	0	0	0	0	0
100.0 μM	NPM	NPM	NPM	NPM	---

TABLE 5-B. Incubation Temperature of 5°C.
Plaque counts per plate for phage Φ6 incubated with glycyrrhizin at 5°C.

PLAQUE COUNT PER PLATE - INCUBATION TEMPERATURE = 5°C

Incubation Concentration of Glycyrrhizin	Plated Concentration of Phage Φ6 $5x10^3$ PFU/ml		$5x10^1$ PFU/ml		Average Normalized to N x 10^3 PFU/ml
	Plates #1	#2	#1	#2	
0.0 μM	>>	800	5	7	667
0.1 μM	0	BP	0	0	0
1.0 μM	0	0	0	0	0
10.0 μM	0	0	0	0	0
100.0 μM	NPM	NPM	NPM	NPM	---

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature as indicated, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml with five respective concentrations of glycyrrhizin.

There were two plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host HB10Y. Two plates were made at each plating concentration. A count of plaques per plate were made. An average of PFU/ml was calculated from both plates and each plating concentration and presented as a value normalized to $Nx10^3$.

TABLE 5-C. Incubation Temperature of 10°C.
Plaque counts per plate for phage Φ6 incubated with glycyrrhizin at 10°C.

PLAQUE COUNT PER PLATE - INCUBATION TEMPERATURE = 10°C					
Incubation Concentration of Glycyrrhizin	Plated Concentration of Phage Φ6 $5x10^3$ PFU/ml		$5x10^1$ PFU/ml		Average Normalized to N x 10^3 PFU/ml
	Plates				
	#1	#2	#1	#2	
0.0 μM	>>>	>>>	15	17	1600
0.1 μM	290	70	0	0	180
1.0 μM	0	0	0	0	0
10.0 μM	0	0	0	0	0
100.0 μM	0	0	0	0	0

TABLE 5-D. Incubation Temperature of 20°C.
Plaque counts per plate for phage Φ6 incubated with glycyrrhizin at 20°C.

PLAQUE COUNT PER PLATE - INCUBATION TEMPERATURE = 20°C					
Incubation Concentration of Glycyrrhizin	Plated Concentration of Phage Φ6 $5x10^3$ PFU/ml		$5x10^1$ PFU/ml		Average Normalized to N x 10^3 PFU/ml
	Plates				
	#1	#2	#1	#2	
0.0 μM	>>>	>>>	69	57	6300
0.1 μM	720	620	9	7	735
1.0 μM	480	310	4	3	372.5
10.0 μM	300	100	0	0	200
100.0 μM	0	0	0	0	0

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature as indicated, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml with five respective concentrations of glycyrrhizin.

There were two plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host HB10Y. Two plates were made at each plating concentration. A count of plaques per plate were made. An average of PFU/ml was calculated from both plates and each plating concentration and presented as a value normalized to $Nx10^3$.

TABLE 5-E. Incubation Temperature of 30°C. Plaque counts per plate for phage Φ6 incubated with glycyrrhizin at 30°C.

PLAQUE COUNT PER PLATE - INCUBATION TEMPERATURE = 30°C					
Incubation Concentration of Glycyrrhizin	Plated Concentration of Phage Φ6 $5x10^3$ PFU/ml		$5x10^1$ PFU/ml		Average Normalized to N x 10^3 PFU/ml
	Plates #1	#2	#1	#2	
0.0 μM	>>>	>>>	44	53	4850
0.1 μM	@1000	@900	8	2	725
1.0 μM	590	610	0	6	450
10.0 μM	190	80	0	0	135
100.0 μM	0	0	0	0	0

TABLE 5-F. Incubation Temperature of 40°C. Plaque counts per plate for phage Φ6 incubated with glycyrrhizin at 40°C.

PLAQUE COUNT PER PLATE - INCUBATION TEMPERATURE = 40°C					
Incubation Concentration of Glycyrrhizin	Plated Concentration of Phage Φ6 $5x10^3$ PFU/ml		$5x10^1$ PFU/ml		Average Normalized to N x 10^3 PFU/ml
	Plates #1	#2	#1	#2	
0.0 μM	>>>	>>>	45	107	7600
0.1 μM	>>>	>>>	40	168	10400
1.0 μM	>>	>>	1	0	50
10.0 μM	40	50	0	0	45
100.0 μM	2	1	0	0	1.5

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature as indicated, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml with five respective concentrations of glycyrrhizin.

There were two plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host HB10Y. Two plates were made at each plating concentration. A count of plaques per plate were made. An average of PFU/ml was calculated from both plates and each plating concentration and presented as a value normalized to $Nx10^3$.

TABLE 5-G. Incubation Temperature of 50°C.
Plaque counts per plate for phage Φ6 incubated with glycyrrhizin at 50°C.

PLAQUE COUNT PER PLATE - INCUBATION TEMPERATURE = 50°C					
Incubation Concentration of Glycyrrhizin	Plated Concentration of Phage Φ6 $5x10^3$ PFU/ml		$5x10^1$ PFU/ml		Average Normalized to N x 10^3 PFU/ml
	Plates #1	#2	#1	#2	
0.0 μM	180	90	2	0	157
0.1 μM	22	5	0	0	13.5
1.0 μM	1	0	0	0	0.5
10.0 μM	0	0	0	0	0
100.0 μM	0	0	0	0	0

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature as indicated, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml with five respective concentrations of glycyrrhizin.

There were two plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host HB10Y. Two plates were made at each plating concentration. A count of plaques per plate were made. An average of PFU/ml was calculated from both plates and each plating concentration and presented as a value normalized to $Nx10^3$.

Data from Control and Normalization Experiments

Each of seven incubation samples were derived from the same phage dilution and incubated at each of seven respective temperatures. Incubation tubes did not contain any glycyrrhizin.

TABLE 6. Temperature Control Experiment Data. Incubation at seven respective temperatures with the incubation concentration of phage for each individual incubation temperature taken from the same stock of phage dilution and no glycyrrhizin.

PLAQUE COUNT PER PLATE from TEMPERATURE CONTROL EXPERIMENT

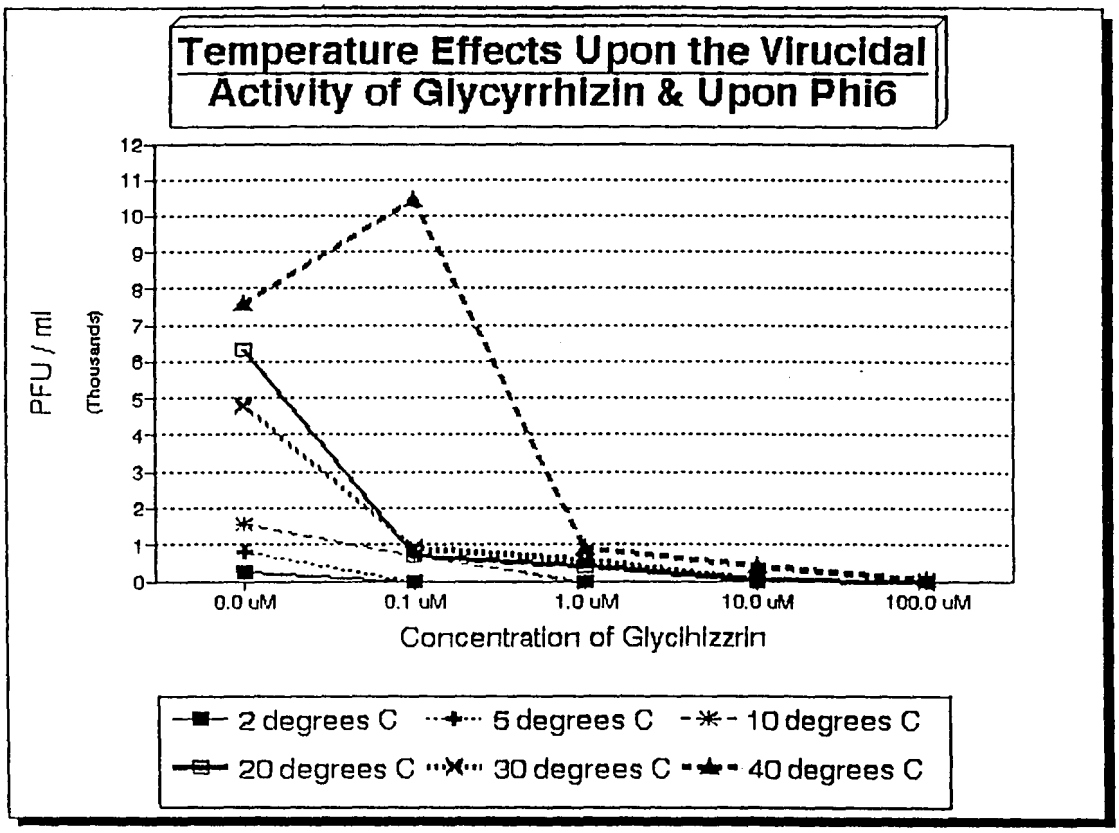
Incubation Temperature	Plated Concentration of Phage Φ6 $5x10^3$ PFU/ml Experimental Run #1	#2	$5x10^1$ PFU/ml Experimental Run #1	#2	Average Normalized to $Nx10^3$ PFU/ml	Average Normalized Percent PFU/ml
2 °C	>>	>>	36	31	3350	62%
5 °C	>>>	>>>	43	39	4100	76%
10 °C	>>>	>>>	53	48	5050	94%
20 °C	>>>	>>>	52	55	5350	100%
30 °C	>>>	>>>	49	46	4750	88%
40 °C	>>	>>	4	5	450	8%
50 °C	@275	@212	2	1	190	3%

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature at each respective temperature indicated, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml with no glycyrrhizin present. To eliminate a variable, the 10ml volume of phage used for incubation, at a concentration of $5x10^5$ PFU/ml was, for each incubation temperature, taken from a single dilution stock prepared at a concentration of $5x10^5$ PFU/ml and at a volume of 200ml. The 10ml of phage was taken from the 200ml stock for each individual incubation temperature and all individual incubations at their respective temperatures were run simultaneously. The same stock was used for both runs of the experiment.

There were two plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host HB10Y. Two experimental runs were made at each plating concentration. A count of plaques per plate were made. An average of PFU/ml was calculated from both runs and each plating concentration and presented as a value normalized to $Nx10^3$. The highest PFU/ml concentration of 5350 x 10^3 PFU/ml occuring at 20°C is established as 100% and used as the denominator to calculate all other percentages indicated.

d.) Graph Section

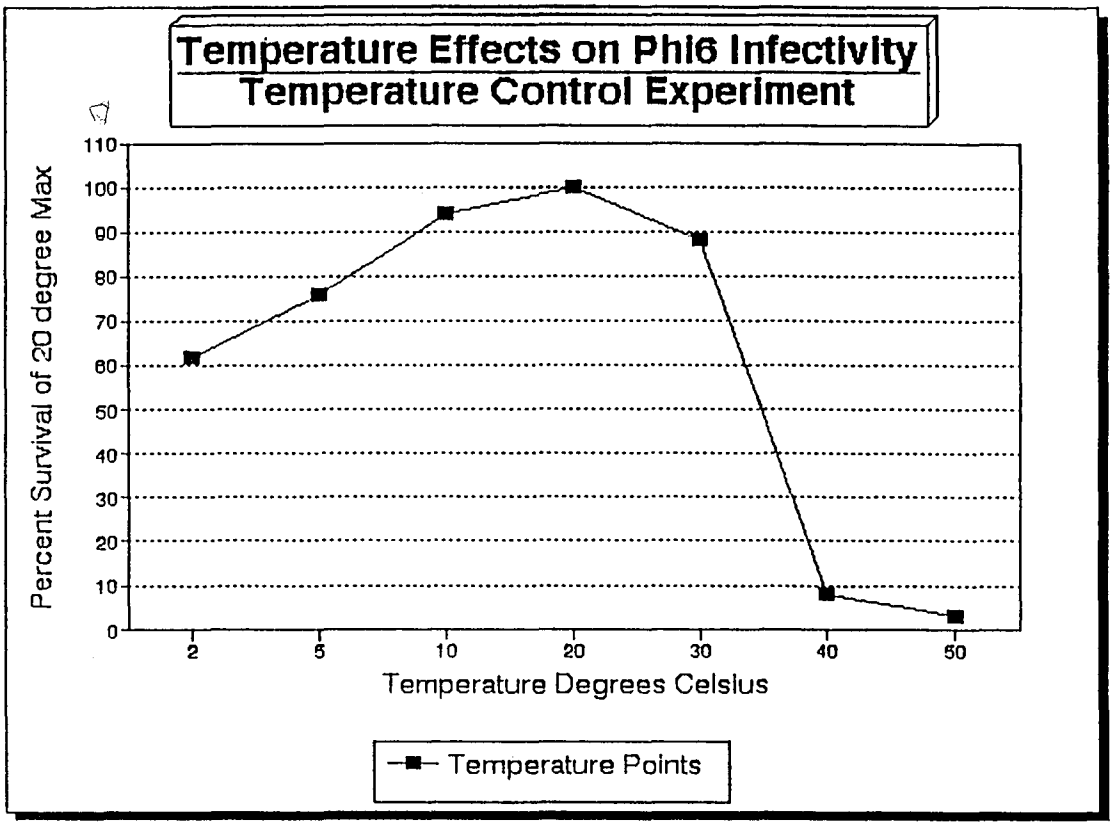
GRAPH 2. Incubation Temperature Effects Upon the Virucidal Activity of Glycyrrhizin and Upon bacteriophage Φ6 infectivity.



The above inactivation curves show PFU/ml as a function of incubation temperature. Data was obtained from using the plaque assay at an incubation time of 30 minutes, incubation temperature at each of seven respective temperatures as indicated, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml with five respective concentrations of glycyrrhizin.

There were two plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host HB10Y. Two plates were made at each plating concentration. A count of plaques per plate were made. An average of PFU/ml was calculated from both plates and each plating concentration and presented as a value normalized to $Nx10^3$. Each data point is an average PFU/ml at a respective incubation temperature and respective incubation concentration of glycyrrhizin. Each curve is at a respective incubation temperature.

GRAPH 3. Incubation Temperature Effects Upon bacteriophage Φ6 infectivity. Temperature Control Experiment.



The above inactivation curves show PFU/ml as a function of incubation temperature. Data was obtained from using the plaque assay at an incubation time of 30 minutes, incubation temperature at each of seven respective temperatures as indicated, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml.

There were two plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml and $5x10^1$ PFU/ml with host HB10Y. Two plates were made at each plating concentration. A count of plaques per plate were made. An average of PFU/ml was calculated from both plates and each plating concentration and presented as a value normalized to $Nx10^3$. Each data point is an average PFU/ml at a respective plated phage concentration and number of plates. Each curve is at a respective incubation temperature.

e.) Results Summary

Without any virucidal compound, phage incubation temperature alone has an effect upon the potential of viral infectivity. Without any virucidal compound, phage incubation temperatures above and below room temperature show a percent inactivation. These results were obtained from the incubation temperature control experiments and these data were used to normalize temperature effects upon phage alone. Letting 100% be set for the highest PFU/ml plaque count, which is room temperature, the drop in plaque forming units can be seen at temperatures above and below room temperature result in the percent inactivation curve shown in Graph No. 3. From Data Graph No. 3, obtained from incubation temperature control results one can see that neither of the two curves above or below room temperature, which show a decrease in plaque formation, are linear. Also the rate of decrease of plaque formation for each of the two curves is not equal. From incubation temperature control results one can see that incubation temperatures above room temperature show a greater reduction in plaque formation to the point of significant virucidal action due to the effects of increased temperature alone (i.e. without any antiviral compound). The effects of temperature on phage alone must be factored out of and used to normalize the experimental results obtained from the individual temperature runs effecting the antiviral activity of glycyrrhizin and phage.

The first thing to observe in Graph No. 2 is that the plaque forming units for 0.0μM of glycyrrhizin all start out at different values. This is simply because each 'per temperature' run of phage concentration for incubation were made separately and at individual times. All 0.0μM values for PFU/ml results are all on the same order, and in fact only differ by a simple factor of n. This is tabularized in the following Table 7.

TABLE 7. Data from Controls in Independent Temperature Runs Plaque-Counts PFU/ml Normalized to a Factor of 'n', whereby n x 10^3; the mantissa is the n-factor and 10^3 is the exponent.

NORMALIZED CONTROL DATA FROM INDEPENDENT TEMPERATURE RUNS	
Temperature	(n-factor) x 10^3
2°C	25
5°C	667
10°C	1600
20°C	6300
30°C	4850
40°C	7600
50°C	157

These control experiments contained no glycyrrhizin and incubation temperature was the only variable. The data in Table 7 were obtained from data in Table 6.

It is these values above that will be used as a normalized standard along with the percentage results found in Table 6 and obtained in the temperature control experiments. Control data from the 20°C Temperature Run is the highest PFU/ml value and is set as the 100% normalized value. All other data points for each Temperature Run are adjusted to the normalization results calculated from the control experiment. Table 8-A and Table 8-B display the calculated steps to obtain the Normalization Multiplier used to normalize Tables 5-A through 5-G into Table 9.

TABLE 8-A. Anticipated n-factor Based on Contol Experiment Results and the Normalization of the Maximum Plaque Count Among all Control Data Values from Each Independent Temperature Run Experiment.

CALCULATIONS TO OBTAIN ANTICIPATED N-FACTOR
FOR EACH TEMPERATURE RUN EXPERIMENT
BASED ON CONTROL EXPERIMENT NORMALIZED PERCENTAGES

Temperature	Average Normalized Percent from the Control Experiment.		Maximum Normalized PFU/ml value out of all the Temperature Runs' Control Data.		Anticipated n-factor for Temperature Run Experiments Based On Control Experiment and Maximum Contol Value from Temp. Runs
2°C	62%	x	6300	=	3900
5°C	76%	x	6300	=	4780
10°C	94%	x	6300	=	5920
20°C	100%	x	6300	=	6300
30°C	88%	x	6300	=	5540
40°C	8%	x	6300	=	500
50°C	3%	x	6300	=	190

Because of the Control Experiment we know the percentage roll-off above and below 20°C. The percentage roll-off is the 'Normalized Percent' obtained from averaging values in the Control Experiment found in Table 6. We also know what Temperature Run Control Data has the highest or maximum PFU/ml value. This maximum PFU/ml value from the controls occurs in the independent 20°C Incubation Temperature Run and is 6300 normalized to the plated concentration of $n \times 10^3$ PFU/ml. If all other variables were equal and held constant between independent temperature runs; then we should obtain the 'Anticipated n-factor' by:

(Anticipated n-factor) = (Normalized Percent) x
(Maximum Temperature Run Control Value)

Table 8-A contains all of the calculated 'Anticipated n-factors'. These values are carried over into Table 8-B and further used to calculate the Normalization Multiplier. The Normalization Multiplier is the value, per each Temperature Run Experiment (i.e. 2°C, 5°C, ..., 50°C), by which all data points in that temperature run are multiplied by to obtain the normalized data table. The Normalized Data Table 9 can then yield percent inactivations which show only temperature effects upon virucidal activity of glycyrrhizin alone.

TABLE 8-B. Normalization Multiplier Calculations Using the result Data From Table 8-A and the Original n-factor from Table 7 we derive the Normalization Multiplier.

CALCULATION OF NORMALIZATION MULTIPLIER
FOR FACTORING OUT TEMPERATURE EFFECTS
FROM ALL TEMPERATURE RUN DATA

Temperature	Anticipated n-factor for Temperature Run Experiments Based On Control Experiment and Maximum Contol Value from Temperature Runs (Table 8-A.)		Original n-factor from each of the Temperature Runs' Control Data (Table 7.) $n \times 10^3$		Normalization Multiplier for removing effects of Temperature from all data in Temp. Runs
2°C	3900	(= / ÷)	25	(x / =)	156.0
5°C	4780	(= / ÷)	667	(x / =)	7.2
10°C	5920	(= / ÷)	1600	(x / =)	3.7
20°C	6300	(= / ÷)	6300	(x / =)	1.0
30°C	5540	(= / ÷)	4850	(x / =)	1.14
40°C	500	(= / ÷)	7600	(x / =)	0.07
50°C	190	(= / ÷)	157	(x / =)	1.2

The 'Normalization Multiplier' is a multiplicand for each independent temperature run that is used to multiply every data point in that temperature run so as to factor out effects of temperature on phage alone and to normalize control data between each individual temperature run. The 'Normalization Multiplier' is obtained by asking what value was the 'Original n-factor' from Temperature Run Control Data multiplied by to obtain the 'Anticipated n-factor' for Temperature Run Control Data'.

k x (Original n-factor) = (Anticipated n-factor)

or by algebraically rearranging terms we obtain:

$$\frac{\text{Anticipated n-factor}}{\text{Original n-factor}} = k;$$

where k is the 'Normalization Multiplier'.

Each Normalization Multiplier is multiplied by averaged values in its respective Temperature Run Table. The Normalization Multiplier for 2°C is 156.0. Hence, 156.0 multiplies each averaged value found in Table 5-A (the 2°C Incubation Temperature Run). The results of this operation

are found in the 2°C column of Table 9. This is done for each temperature.

For example, all the results for Table 5-A, 2°C incubation temperature, will be multiplied by 156 to normalize out phage incubation concentration differences existing between each independent temperature run and to factor out effects of temperature upon phage alone (no glycyrrhizin). These adjustments result in normalized data values for each data point arising from seven temperatures and five concentrations of glycyrrhizin. These are displayed in Table 9. In addition Table 9 contains percent inactivation values obtainable from these normalizations.

TABLE 9. Normalized Temperature Effects upon Virucidal Activity of Glycyrrhizin upon Phage Φ6

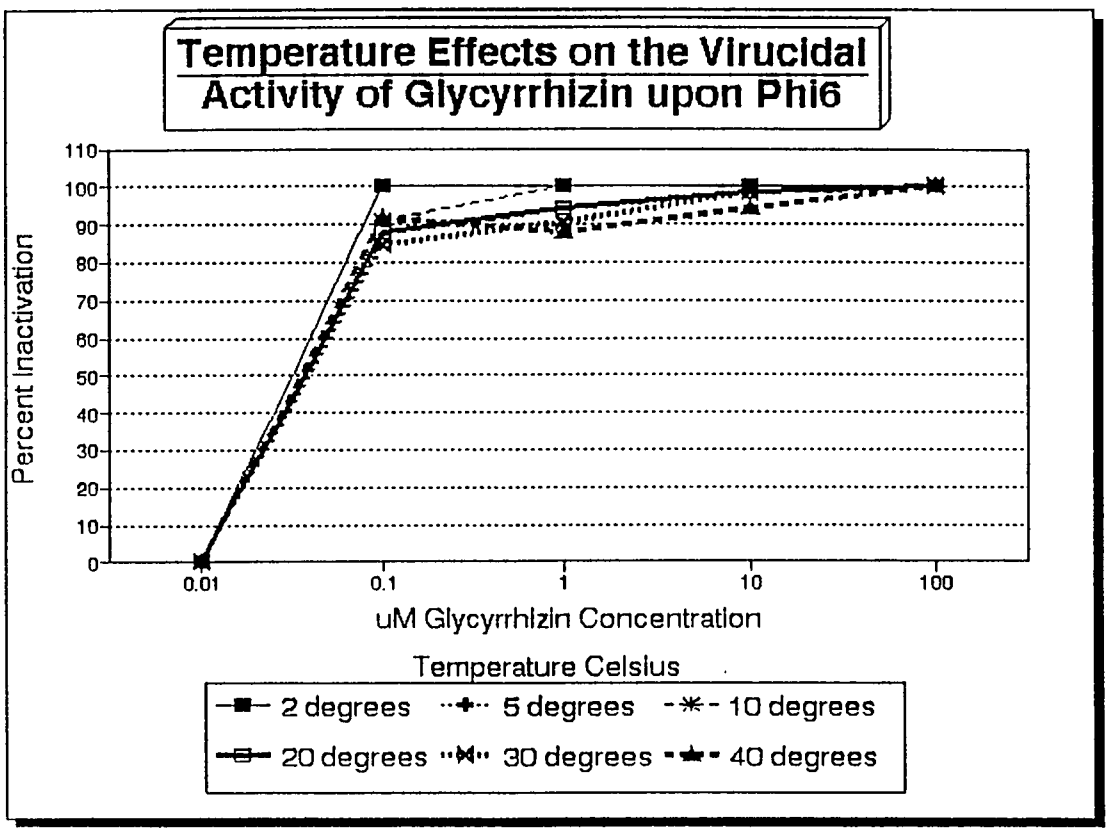
Table 9 combines all data normalized from each independent Temperature Run Data Table 5-A through 5-G by multiplying the 'Normalization Multiplier' found in Table 8, for each respective temperature, by each averaged plaque count in each Table 5-A to 5-G for each concentration of glycyrrhizin. All plaque values have been normalized to $n \times 10^1$ PFU/ml.

AVERAGED AND NORMALIZED PLAQUE COUNT PER PLATE AND PERCENT INACTIVATION

Glycyrrhizin Incubation Concentration	TEMPERATURE RANGE 2°C	5°C	10°C	20°C	30°C	40°C	50°C
0.0μM	39.0	47.8	59.2	63.0	55.4	5.0	1.9
%Inactivation	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
0.1μM	0.0	0.0	5.3	7.4	8.27	7.3	0.162
%Inactivation	0.0%	100.0%	91.0%	88.2%	85.0%	---	91.4%
1.0μM	0.0	0.0	0.0	3.7	5.13	0.6	0.0
%Inactivation	100.0%	100.0%	100.0%	94.1%	90.7%	88.0%	100.0%
10.0μM	0.0	0.0	0.0	1.0	0.91	0.3	0.0
%Inactivation	100.0%	100.0%	100.0%	98.4%	98.3%	94.0%	100.0%
100.0μM	NPM	NPM	0.0	0.0	0.0	0.01	0.0
%Inactivation			100.0%	100.0%	100.0%	99.76%	100.0%

Table 9 combines all data normalized from each independent Temperature Run Data Table 5-A through 5-G by multiplying the 'Normalization Multiplier' found in Table 8, for each respective temperature, by each averaged plaque count in each Table 5-A to 5-G for each concentration of glycyrrhizin. All plaque values have been normalized to $n \times 10^1$ PFU/ml.

Graph 4. Normalized Temperature Effects upon Virucidal Activity of Glycyrrhizin upon Phage ϕ6



Graph 4 displays the normalized percent inactivation curve for each independent temperature experiment. The plaque assay was performed under seven different incubation temperatures. Data for each curve has been normalized so as to factor out temperature effects on phage during incubation and any variable differences between phage concentrations in each temperature run. Data for the graph was obtained from Table 9.

f.) Conclusions and Discussion

The effects of temperature on both glycyrrhizin and Φ6 can are seen in the Graph 2. The effects of temperature upon Φ6 alone (i.e. no glycyrrhizin) can be seen clearly in Graph 3. Looking at Graph 3 it is seen that at temperatures above and below 20°C the PFU/ml plaque count drops. The number of plaques decreases more rapidly at temperatures above 20°C than those below 20°C. Most likely the higher temperatures have more of a virucidal effect whereas the results below 20°C display more of a reduced infectivity potential while maintained at the lower temperatures. Recall that Φ6 phage is stored long term (4-6 months) at 2°C. All room temperature inactivation experiments, allowed incubation tubes, containing phage, to first reach and sit at room temperature prior to commencement of incubation and certainly therfore prior to actual plating. Host infection takes place during plating. It is during plating that host and phage are vortexed together in the cooling agar. Obviously virus is not inactivated at lower temperatures but has a reduced potential for infectivity when incubated and then immediatly plated while at the lower temperatures. Bacteriophage Φ6 is however partially inactivated when incubated at the higher temperatures. Experimental data, which are not included here, were obtained to allow phage incubated at higher temperatures to cool down to and sit at room temperature.

At various intervals of cooling sample plates where made. After reaching room temperature sample plates where made at six 15 minute intervals in order to observe if any plaque forming units increase. The results of these experiments showed that there was no appreaciable increase in viral infectivity or plaque forming units as a result of allowing incubated phage to cool to room temperature and even sit at room temperature for 2-3 hours.

After the normalization results of Table 4 (Graph 3) are applied to temperature data of Table 3 (Graph 2) and all effects of temperature acting on Φ6 are normalized out then the effects of temperature on glycyrrhizin virucidal activity can be observed. The calculations from the results section yield the Table 9 and Graph 4. One can see that the virucidal activit of glycyrrhizin is not appreciably affected by temperature changes within a range from 2°C to 50°C. The incubation temperatures below 20°C however, show a slightly higher percent inactivation at 0.1μM of glycyrrhizin. There is a peculiar data point in all graphs occuring at 0.1 μM of glycyrrhizin for the 40°C run, a temperature above 20°C, where percent inactivation still remains at approximately 0%. Several experiments were peformed above and below the 0.1μM concentration of glycyrrhizin. Inactivation results fluctuate around the 0.1μM data point. This is because of the slope of the

percent inactivation curve typical in this region of a sigmoidal curve. The percent inactivation of Φ6 by glycyrrhizin increases very slightly as incubation temperature decreases at the data point 0.1μM. This is only due to fluctuation errors about this data point and not actual effects of temperature on glycyrrhizin at this data point. Temperature has more of an effect on Φ6 directly than on glycyrrhizin virucidal activity. Temperature has no appreciable effect on glycyrrhizin activity.

All data points involving 0.1 μM glycyrrhizin for 40°C do not show the consistent percent inactivation as seen at all other temperatures. This could be an interesting phenomenon or simply an experimental error. Repeat experiments, as stated above, would occasionally show similar fluctuations at various temperatures including 20°C. This phenomenon, if not characteristic of the sigmoidal curve in this region, is then most likely due to an error in lab technique. There may be some indication of intermitant activity at or below some critical level such as 0.1μM of glycyrrhizin.

This phenomenon might become more evident or occur more regularly at higher temperatures. Since, however, the virucidal effects of temperature are very prevalent at 50°C, temperatures above 50°C would outweigh virucidal effects of

glycyrrhizin.

In summary the expected effects of temperature do occur. Results show that just incubating phage alone, at temperatures above and below room temperature, have an effect on phage infectivity. After effects of temperature on phage are factored out, one can see that changes in incubation temperature have no appreciable effect on the virucidal activity of glycyrrhizin upon Φ6. Glycyrrhizin maintains a relatively consistant inactivation curve through a temperature range between 2°C and 50°C.

3. Effects of Exogenous Lipids upon the Virucidal Activity of Glycyrrhizin upon Φ6

a.) Rationale and Hypothesis

In an *in-vivo* system the activity of a potential antiviral compound becomes more complex. The interaction of numerous molecules increase the number of variables that affect, in a positive or negative way, the potential of an antiviral compound. In an attempt to be able to measure and control individual variables and yet add one degree of complexity, individual exogenous lipids were added and present in incubation tubes containing phage before the time of adding and incubating with the virucidal compound glycyrrhizin. Effects due to the addition of a single exogenous lipid were measured. Six different exogenous lipids were tested. Previous work investigating interference effects on virucide activity had shown that certain fatty acids and in general exogenous lipids could interfere with a virucidal compound (20). Certain virally noninactivating and noninhibiting fatty acids were potent interfering agents (20). I am postulating here that even inactivating fatty acids could interfere with another virucidal agent, therefore exogenous lipids were selected for interference study in this research. I had hypothesized that the exogenous lipids would have definite effects on glycyrrhizin. I had mostly expected that these lipids would interfere and reduce the virucidal activity of glycyrrhizin. Individual lipids that did interefere could implicate

mechanisms whereby the presence of other molecules add a modifying variable to the effectiveness of a virucidal agent.

b.) Experimental Design and Method

All experiments were performed at room temperature. The incubation concentration for all experiments was 5 x 10^5 PFU/ml and the plated concentrations of phage was 5 x 10^2, 5x 10^1 PFU/ml and 5x 10^0 PFU/ml. Phage were added to incubation tubes first. Each respective exogenous lipids was then added to the incubation tubes and heavily vortexed at each of the respective concentrations. Incubation tubes were allowed to sit for 30-45 seconds before adding glycyrrhizin. The virucidal compound, glycyrrhizin was added to the incubation tubes at a constant concentration of 10μM and then vortexed. Time t=0 began immediatly after the glycyrrhizin was added and vortexed. The incubation time, It, was 30 minutes. A single run was made for each exogenous lipid. Two plates per plating concentration were made. The control plates contained only phage. Neither the respective exogenous lipid nor virucidal compound were added to the control plates. All incubation tubes were made from equal 10ml volumes from the same stock concentration of phage. This was done so that each incubation sample as well as controls came from the same dilution lineage. Three exogenous lipid runs were performed at the same time and from the same phage stock. An average was taken between the first set of three and an average for the second set of three exogenous lipids controls to yield a more accurate

control value. These two averages yielded a ratio for deriving a complete table of percent inactivation based on 0.0 μM of the exogenous lipid and 10.0μM of the antiviral compound glycyrrhizin.

c.) Data Section

TABLE 10-A. Phosphatidyl Choline Effects on Glycyrrhizin
Effects of Phosphatidyl Choline on the Virucidal Activity of Glycyrrhizin upon phage Φ6.

PLAQUES PER PLATE - PHOSPHATIDYL CHOLINE							
Incubation Concentration of Phosphatidyl Choline	PLATED CONCENTRATIONS (PFU/ml)						Average Plaque Count Normalized to $nx10^0$ PFU/ml
	$5x10^3$		$5x10^1$		$5x10^0$		
	PLATES						
	1	2	1	2	1	2	
CONTROL	>>	>>	74	61	8	11	8.12
0.0μM	71	82	1	0	0	0	0.084
1.0μM	650	700	8	6	0	0	0.688
10.0μM	>>	>>	66	91	5	5	6.43
100.0μM	>>	>>	82	105	4	5	7.00

Control plates contained 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin.

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, incubation concentration of glycyrrhizin of 10μM except for control plate, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml.

There were three plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml, $5x10^1$ PFU/ml, and $5x10^0$ PFU/ml with host HB10Y. Two plates at each concentration were made. There were 4 concentrations of the respective exogenous lipid. A count of plaques per plate was made. An average was calculated from plaque count data from all plated concentrations and both plates and normalized to the $nx10^0$ PFU/ml plate count.

TABLE 10-B. Arachidylic Acid Effects on Glycyrrhizin
Effects of Arachidylic Acid on the Virucidal Activity of Glycyrrhizin upon phage Φ6.

PLAQUES PER PLATE - ARACHIDYLIC ACID							
Incubation Concentration of Arachidylic Acid	PLATED CONCENTRATIONS (PFU/ml)						Average Plaque Count Normalized to $nx10^0$ PFU/ml
	$5x10^3$		$5x10^1$		$5x10^0$		
	PLATES						
	1	2	1	2	1	2	
CONTROL	>>>	>>>	101	69	8	6	7.75
0.0μM	80	65	1	0	0	0	0.082
1.0μM	>>	>>	11	9	1	1	1.0
10.0μM	>>>	>>>	97	94	10	8	9.27
100.0μM	∞	∞	115	85	10	17	11.75

Control plates contained 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin.

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, incubation concentration of glycyrrhizin of 10μM except for control plate, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml.

There were three plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml, $5x10^1$ PFU/ml, and $5x10^0$ PFU/ml with host HB10Y. Two plates at each concentration were made. There were 4 concentrations of the respective exogenous lipid. A count of plaques per plate was made. An average was calculated from plaque count data from all plated concentrations and both plates and normalized to the $nx10^0$ PFU/ml plate count.
LH8

TABLE 10-C. Cholesterol Effects on Glycyrrhizin
Effects of Cholesterol on the Virucidal Activity of Glycyrrhizin upon phage Φ6.

PLAQUES PER PLATE - CHOLESTEROL							
Incubation Concentration of Cholesterol	PLATED CONCENTRATIONS (PFU/ml)						Average Plaque Count Normalized to $nx10^0$ PFU/ml
	$5x10^3$		$5x10^1$		$5x10^0$		
	PLATES						
	1	2	1	2	1	2	
CONTROL	>>>	>>>	128	115	13	9	11.58
0.0μM	110	106	1	0	0	0	0.105
1.0μM	770	580	7	4	0	0	0.613
10.0μM	>>	>>	19	11	0	0	1.5
100.0μM	>>>	>>>	41	24	0	0	3.25

Control plates contained 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin.

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, incubation concentration of glycyrrhizin of 10μM except for control plate, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml.

There were three plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml, $5x10^1$ PFU/ml, and $5x10^0$ PFU/ml with host HB10Y. Two plates at each concentration were made. There were 4 concentrations of the respective exogenous lipid. A count of plaques per plate was made. An average was calculated from plaque count data from all plated concentrations and both plates and normalized to the $nx10^0$ PFU/ml plate count.
LH8

TABLE 10-D. **Stearic Acid Effects on Glycyrrhizin**
Effects of Stearic Acid on the Virucidal Activity of Glycyrrhizin upon phage Φ6.

PLAQUES PER PLATE - STEARIC ACID							
Incubation Concentration of Stearic Acid	PLATED CONCENTRATIONS (PFU/ml)						Average Plaque Count Normalized to $nx10^0$ PFU/ml
	$5x10^3$		$5x10^1$		$5x10^0$		
	PLATES						
	1	2	1	2	1	2	
CONTROL	>>>	>>>	55	68	6	5	5.9
0.0μM	65	71	0	0	0	0	0.068
1.0μM	1	2	0	0	0	0	0.0015
10.0μM	0	0	0	0	0	0	0.0
100.0μM	NHL	NHL	BP	NHL	0	0	---

Control plates contained 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin.

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, incubation concentration of glycyrrhizin of 10μM except for control plate, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml.

There were three plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml, $5x10^1$ PFU/ml, and $5x10^0$ PFU/ml with host HB10Y. Two plates at each concentration were made. There were 4 concentrations of the respective exogenous lipid. A count of plaques per plate was made. An average was calculated from plaque count data from all plated concentrations and both plates and normalized to the $nx10^0$ PFU/ml plate count.

TABLE 10-E. **Oleic Acid Effects on Glycyrrhizin**
Effects of Oleic Acid on the Virucidal Activity of Glycyrrhizin upon phage Φ6.

PLAQUES PER PLATE - OLEIC ACID							
Incubation Concentration of Oleic Acid	PLATED CONCENTRATIONS (PFU/ml)						Average Plaque Count Normalized to $nx10^0$ PFU/ml
	$5x10^3$		$5x10^1$		$5x10^0$		
	PLATES						
	1	2	1	2	1	2	
CONTROL	>>>	>>>	49	58	6	6	5.8
0.0μM	69	44	0	0	0	0	0.057
1.0μM	410	350	8	7	0	0	0.565
10.0μM	2	3	0	0	0	0	0.0025
100.0μM	NHL	NHL	0	0	0	0	---

Control plates contained 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin.

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, incubation concentration of glycyrrhizin of 10μM except for control plate, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml.

There were three plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml, $5x10^1$ PFU/ml, and $5x10^0$ PFU/ml with host HB10Y. Two plates at each concentration were made. There were 4 concentrations of the respective exogenous lipid. A count of plaques per plate was made. An average was calculated from plaque count data from all plated concentrations and both plates and normalized to the $nx10^0$ PFU/ml plate count.
LH8

TABLE 10-F. **Caprylic Acid Effects on Glycyrrhizin**
Effects of Caprylic Acid on the Virucidal Activity of Glycyrrhizin upon phage Φ6.

PLAQUES PER PLATE - CAPRYLIC ACID							
Incubation Concentration of Caprylic Acid	PLATED CONCENTRATIONS (PFU/ml)						Average Plaque Count Normalized to $nx10^0$ PFU/ml
	$5x10^3$		$5x10^1$		$5x10^0$		
	PLATES						
	1	2	1	2	1	2	
CONTROL	>>>	>>>	46	44	4	7	4.71
0.0μM	41	83	0	0	0	0	0.062
1.0μM	10	17	0	0	0	0	0.0135
10.0μM	0	0	0	0	0	0	0.0
100.0μM	NHL	NHL	BP	NHL	0	0	---

Control plates contained 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin.

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, incubation concentration of glycyrrhizin of 10μM except for control plate, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml.

There were three plated concentrations of phage Φ6 namely, $5x10^3$ PFU/ml, $5x10^1$ PFU/ml, and $5x10^0$ PFU/ml with host HB10Y. Two plates at each concentration were made. There were 4 concentrations of the respective exogenous lipid. A count of plaques per plate was made. An average was calculated from plaque count data from all plated concentrations and both plates and normalized to the $nx10^0$ PFU/ml plate count.

TABLE 11. Effects of Exogenous Lipids on Virucidal Activity of Glycyrrhizin upon phage Φ6 Expressed as a Percent Inactivation

	PERCENT INACTIVATION					
	EXOGENOUS LIPID					
Incubation Concentration	$PC_{(1)}$	Arachidylic Acid	Cholesterol	Stearic Acid	Oleic Acid	Caprylic Acid
CONTROL	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
0.0μM	99.0%	99.0%	99.0%	98.86%	98.86%	98.86%
1.0μM	92.5%	89.1%	93.3%	99.9%	89.7%	99.8%
10 μM	39.6%	-1.4%	83.6%	100%	99.9%	100%
100μM	22.5%	-28.6%	64.4%	---	---	---

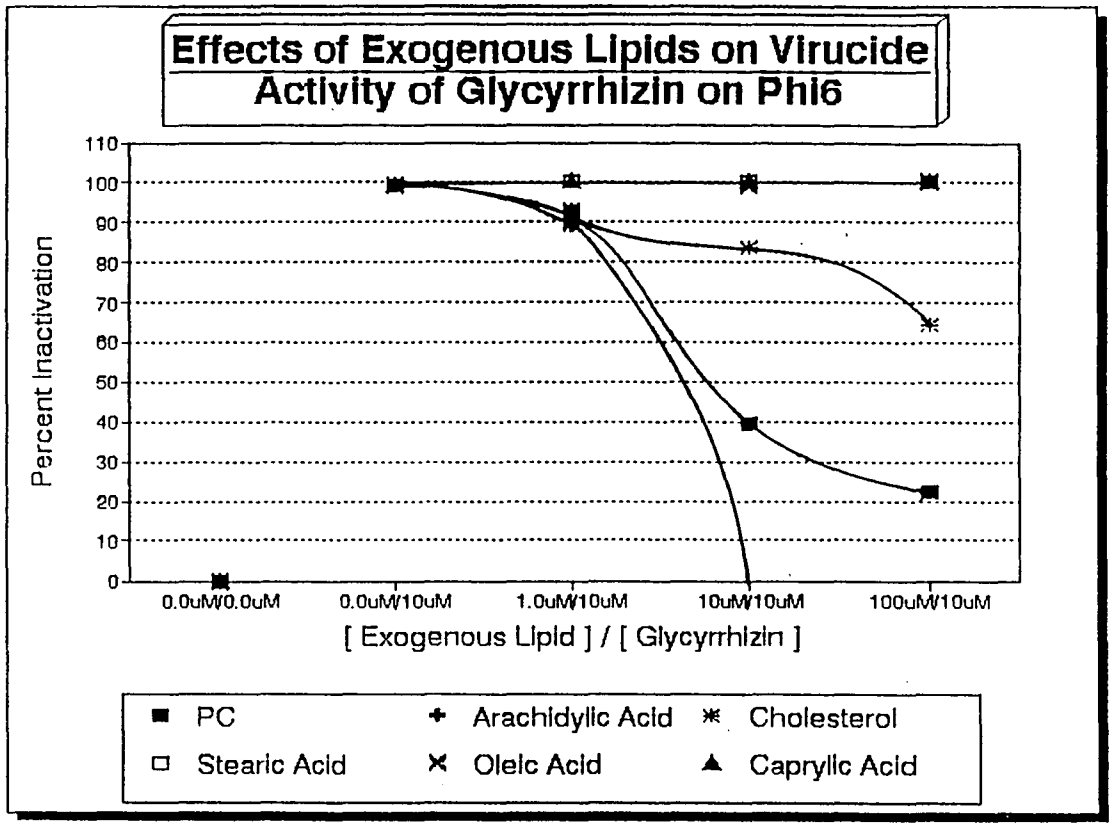
Control plates contained 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin.

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, incubation concentration of glycyrrhizin of 10μM except for control plate, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml. There were four concentrations of each of the respective exogenous lipid namely; 0.0μM, 1.0μM, 10.0μM, and 100.0μM. Data used to derive percent inactivation values were obtained from Tables 10-A through 10-F.

(1) PC is Phosphatidyl Choline.

d.) Graph Section

GRAPH 5. Effects of Exogenous Lipids on Virucidal Activity of Glycyrrhizin upon phage Φ6 Expressed as a Change in Percent Inactivation



Concentration values of 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin were used as controls.

Data for the above graph was taken from Table 11. Data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, incubation concentration of glycyrrhizin of 10μM except for control plate, and a phage Φ6 incubation concentration of $5x10^5$ PFU/ml. There were four concentrations of each of the respective exogenous lipid namely; 0.0μM, 1.0μM, 10.0μM, and 100.0μM. Data used to derive percent inactivation values in Table 11 were obtained from Tables 10-A through 10-F.

(1) PC is Phosphatidyl Choline.

e.) Results Summary

Phosphatidyl choline, arachidylic acid, and cholesterol show virucidal neutralizing effects upon glycyrrhizin.

Stearic acid, oleic acid, caprylic acid show no appreciable virucidal neutralizing effects upon glycyrrhizin.

Arachidylic acid showed the most effective virucidal neutralizing effect on the virucidal compound glycyrrhizin of all the lipids tested.

Virucidal neutralizing effects for phosphatidyl choline, arachidylic acid, cholesterol and stearic acid begin at 1.0μM concentrations. Virucidal neutralizing effects sharply increase for arachidylic acid immediately after concentrations of 1.0μM. Virucidal neutralizing effects of oleic acid only appear slightly at a concentration of 1.0μM.

There is no observable virucidal neutralizing effects for neither stearic acid nor caprylic acid at any concentrations tested. Phosphatidyl choline shows the second most effective and cholesterol the third most effective virucidal neutralizing effect on the virucidal

compound glycyrrhizin of all the lipids tested.

It has not been fully investigated here, but there is some indication that stearic acid, caprylic acid and especially oleic acid were showing, instead of a neutralizing effect upon glycyrrhizin, a possible slight virucidal effect in addition to the virucidal activity of glycyrrhizin. The higher concentrations of stearic acid, oleic acid and caprylic acid are most likely having a cytotoxic effect because the higher concentrations of these lipids consistently either inhibited growth or exhibited abnormal growth of host lawn. These higher incubation concentrations will yield higher concentrations in culture.

f.) Conclusions and Discussion

The effects of phosphatidyl choline, arachidylic acid, and cholesterol upon the virucidal activity of glycyrrhizin has been termed in a general sense as 'virucidal neutralizing'. These compounds are more specifically and most likely providing a protective action against glycyrrhizin especially because they are present in incubation tubes before glycyrrhizin is added. I do not believe that there is any enzymatic chemistry taking place between these protective lipids and glycyrrhizin but rather molecular interactions.

Since it has already been shown that the virucidal activity of glycyrrhizin would occur without any lipid present, virucidal activity would therefore certainly occur prior to the addition of lipid. Consequently the lipid would need to be present in the incubation tube prior to adding glycyrrhizin.

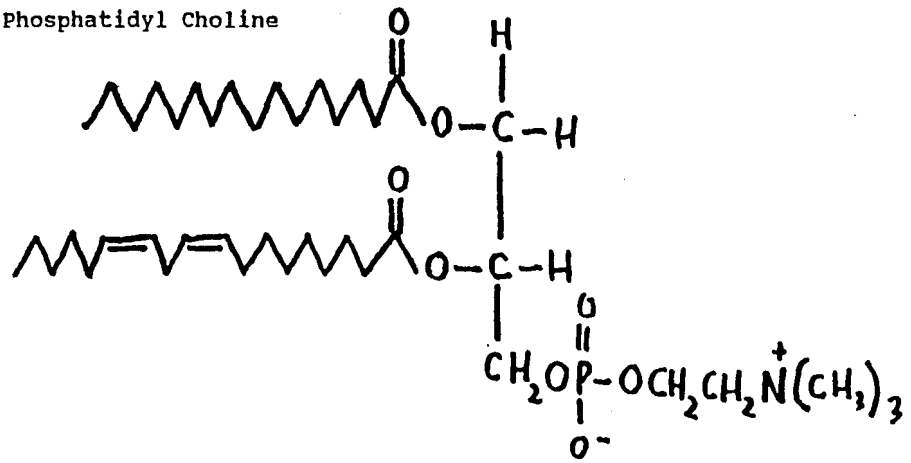
In attempting to identify areas of further and follow-up research, I would find it interesting to perform an experiment that has a control with phage but no glycyrrhizin and no lipid and a second incubation tube where at time t=0 only phage and glycyrrhizin are present. At time t=15 minutes, lipid is added to the second tube for the remaining

15 minutes. Plates would be made from both tubes at time t=30 minutes. A third plate would be made from the first control tube. The top agar tube for the third plate ($t=30min^{+}$) would contain lipid along with the host and top agar and has been vortexed prior to adding phage. Once phage is added the agar tube would then be vortexed and immediately plated. A third tube would contain lipid and glycyrrhizin and would incubate for 30 minutes. Phage would be added at time t=30 minutes, immediately vortexed, diluted and plated. As done in this research phage and lipid would be present in a fourth incubation tube before adding glycyrrhizin ($t=0^{-}$). Total incubation time for all incubation tubes would be 30 minutes at which point plates for each incubation tube would be made. Plates would also be made from all incubation tubes at t=14 minutes and t=29 minutes just prior to the addition of lipid to tube 2 and phage to the lipid-host-top-agar tube. This would provide further evidence if neutralizing effects of these lipids are protective, arresting or both and to what degree of either.

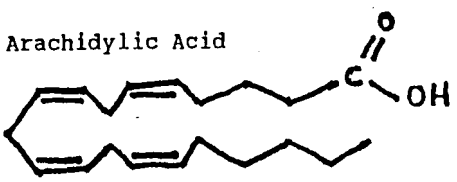
Before speculating on the molecular interactions of lipid and glycyrrhizin, which may be responsible for the protective effects of the lipid, the following molecular structures for phosphatidyl choline, arachidylic acid, cholesterol and again glycyrrhizin are shown for comparison in Diagram 4.

Diagram 5. Molecular structure of protective exogenous lipids used-in this section of this reasearch.

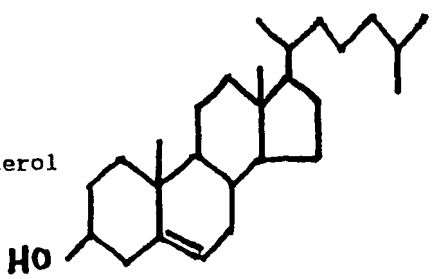
Phosphatidyl Choline



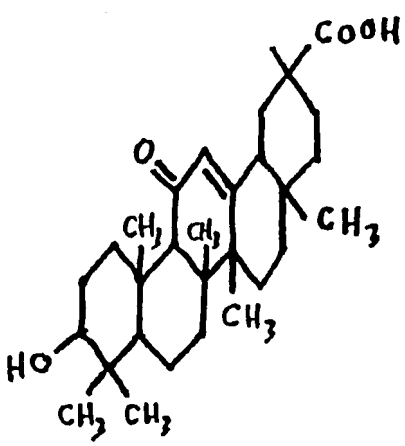
Arachidylic Acid



Cholesterol



Glycyrrhizin



An important question to ask is: are there any common molecular characteristics that allow these three molecules to interact with glycyrrhizin and thereby provide a virucidal protective mechanism? If so, what are the possible common motifs?

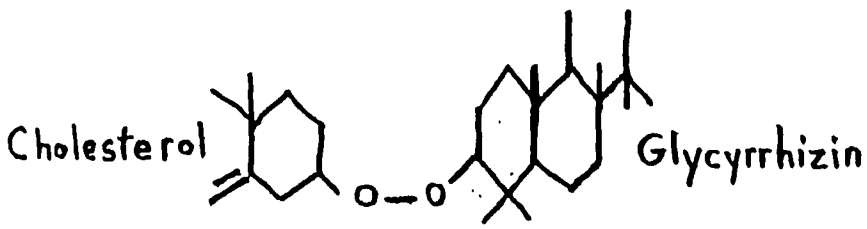
Arachidylic acid, cholesterol and glycyrrhizin all have an OH group off of a carbon molecule. The OH group off of the carbon molecule of glycyrrhizin is involved in the glycoside linkage to sugars. In the glycoside form the gelling properties of glycyrrhizin are very apparent. The glycoside form of glycyrrhizin naturally occurs in its plant source.

Recall that the lipid envelope of the phage Φ6 contains PG which is also a phosphatide as is PC. This fact may suggest some similar interactions between glycyrrhizin with the phage envelope and glycyrrhizin with the protective mechanisms of PC.

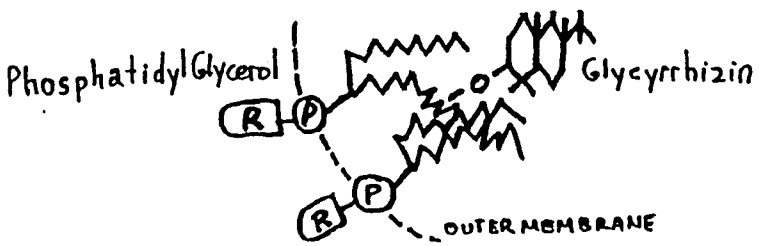
Glycyrrhizin may be interacting with a given lipid by way of one of the following speculative models: molecule :: molecule interaction; lipid-micelle::glycyrrhizin-attachment; or amorphous aggregations of lipids, medium salts, and glycyrrhizin. Each individual lipid may have a different method of interaction.

Diagram 6. Speculative Models of Exogenous Lipid and Glycyrrhizin Interaction.

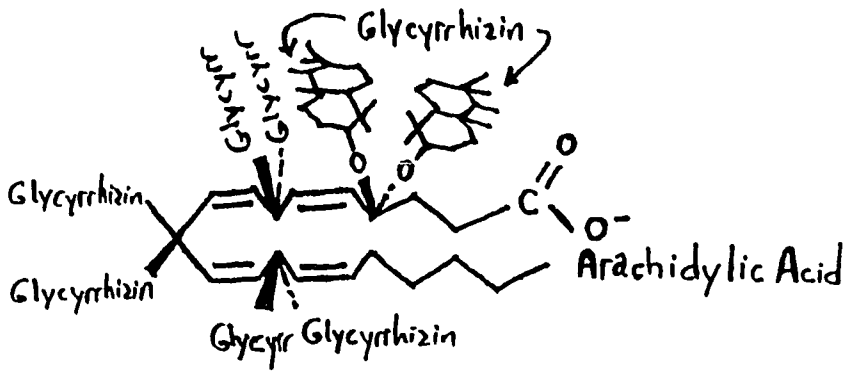
Lipid molecule to Glycyrrhizin Molecule Interaction



Lipid Micelle Formation and Glycyrrhizin Surface Attachment



Lipid and Glycyrrhizin Molecule Aggregations



A peculiar result is that arachidylic acid is among the most active of virucidal neutralizing lipids. Cholesterol is somewhat similar in structure and size to glycyrrhizin and may form a semi-semetrically sized structure about a bond between hydroxal groups that each molecule contains. The neutralizing lipid PC is similar to the the viral lipid envelope PG. Speculating that glycyrrhizin is interacting with PG of the phage envelope, and that PG might be involved in events that lead to P3 dislodgement during phage inactivation, I would postulate that glycyrrhizin may interact with PC and reduce its involvement with PG in the viral envelope and hence reduce the virucidal activity of glycyrrhizin. Arachidylic acid is different than PG and chosterol and more like the other lipids tested which did not show a great neutralizing or protective level against the virucidal agent glycyrrhizin. One characteristic of arachidylic acid, which might contribute to its virucidal neutralizing ability and does distinguish its structure from the other lipids tested is that it does have 4 unsaturated bonds along with its characteristic fatty acid tail.

The interaction of glycyrrhizin with each of these lipids may be different. PC may interact by micelle formation; arachidylic acid may interact by glycoside linkages; and cholesterol may interact by aggregation. All these interactions may involve or require certain factors in

the host medium or other medium properties such as ions, pH and temperature.

Another possible mechanism may involve the interaction of these lipids with the surface of phage Φ6 in such a way as to ultimately protect P3 and P6 envelope protein from glycyrrhizin. This possible mechanism includes lipid molecules blocking and thereby protecting the area of the Φ6 attachment protein from glycyrrhizin. Another possible mechanism is that the protective lipids attract glycyrrhizin away from the Φ6 surface proteins dissallowing glycyrrhizin to interact. The interaction of protective lipid and glycyrrhizin may not be by way of a mutual molecular bond. A chemical action upon an active site of glycyrrhizin may be occuring.

The protective lipids may be interacting with the phage envelope and thereby reduce the fluidity of the lipid envelope. This reduction in the fluidity of the lipid membrane of the phage may prevent further intercalation or interaction by glycyrrhizin. The lipid envelope of the phage may have a higher affinity for this affect by the exogenous lipid than the glycyrrhizin. This affect may be stablized by the pre-exposure to the protective lipids before glycyrrhizin.

By whatever mechanism that these lipids inhibit the virucidal activity of glycyrrhizin, these mechanisms do not completely disrupt or interfere with viral infectivity.

In the next section several incubation experiments each with a different concentration of glycyrrhizin and a constant concentration of each individual protective lipid are performed. Follow-up research should investigate several concentrations of each individual lipid with several concentrations of glycyrrhizin for each lipid concentrations in both host medium (which contains salts, nutrients, etc..) and in distilled water as the incubation solvent. By performing these experiments the neutralizing ratios may be better established and would also determine if any factors are contributed by host growth medium. Experiments with only phage and exogenous lipid would allow the determination of virucidal activity of any of these exogenous lipids.

4. The effects of increased concentrations of glycyrrhizin in the presence of a protective level of an exogenous lipid.

a.) Rationale and Hypothesis

In the previous set of experiments I investigated the effects of six exogenous lipids and their effects upon the virucidal activity of glycyrrhizin at 10μM. In my results I noted that three of the six exogenous lipids did show protective or neutralizing effects upon glycyrrhizin. I also observed from those results at what concentrations each of the effective neutralizing exogenous lipids began to show an appreciable effect. In this set of experiments I have selected and held constant the concentration of the exogenous lipids, where appreciable neutralizing activity was observed, while increasing the concentration of the virucidal compound glycyrrhizin in order to determine possible recovery of a virucidal activity. I had hypothesized that increasing concentrations of glycyrrhizin would overcome the protective effects of the exogenous lipids. I also wanted to determine if a concentration ratio between lipid and glycyrrhizin existed where 50% viral inactivation occurs. This parameter would indicate a critical concentration ratio or critical IRa/n value. Recall that IRa/n is the incubation ratio of an antiviral concentration to an 'antiviral neutralizing' concentration. The critical IRa/n would be where virucidal activity is reduced by 50%.

b.) Experimental Design and Method

First, 160 ml of 5x 10^5 PFU/ml phage solution is prepared. The 160ml is divided eqaully into 16 sterile incubation tubes containing 10ml each which still is at a phage concentration of 5 x 10^5 PFU/ml.

A 4x4 matrix of incubation tubes is setup and prepared to contain the following final additives:

Tube #1	Tube #2	Tube #3	Tube #4
0.0μM Glyz.	0.0μM Glyz.	100μM Glyz.	1mM Glyz
No lipd	No Lipid	No Lipid	No Lipid
5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6

Tube #5	Tube #6	Tube #7	Tube #8
0.0μM Glyz.	10μM Glyz.	100μM Glyz.	1mM Glyz
5.0 μM EL1	5.0 μM EL1	5.0 μM EL1	5.0 μM EL1
5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6

Tube #9	Tube #10	Tube #11	Tube #12
0.0μM Glyz.	10μM Glyz.	100μM Glyz.	1mM Glyz
5.0 μM EL2	5.0 μM EL2	5.0 μM EL2	5.0 μM EL2
5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6

Tube #13	Tube #14	Tube #15	Tube #16
0.0μM Glyz.	10μM Glyz.	100μM Glyz.	1mM Glyz
5.0 μM EL3	5.0 μM EL3	5.0 μM EL3	5.0 μM EL3
5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6	5x10^5 PFU/ml Φ6

10ml of 5 x 10^5 PFU/ml of phage is ready in incubation tubes. At time t = -1 minute, the respective exogenous lipid is added to its tube and heavily vortexed. Time, t=0, begins after the respective concentration of glycyrrhizin is added to the proper tube and vortexed. The time, t=0, is staggered by 10 minutes along each of 16 incubation tubes to allow time for plating. At time, t = 30 minutes, three dilutions are made from each of 16 incubation tubes. These three dilutions correspond to plating concentrations of 5 x 10^2 PFU/ml, 5 x 10^1 PFU/ml and 5 x 10^0 PFU/ml of the original dilution lineage. Two plates are made from each of the three plating concentrations, hence a total of six plates per incubation tube; two at each plating concentration to allow a wider and accurate range of plaque counting.

The control for this entire experiment is incubation tube #1.

c.) Data Section

TABLE 12. Summary of the effects of increased concentrations of glycyrrhizin in the presence of a protective level of an exogenous lipid.

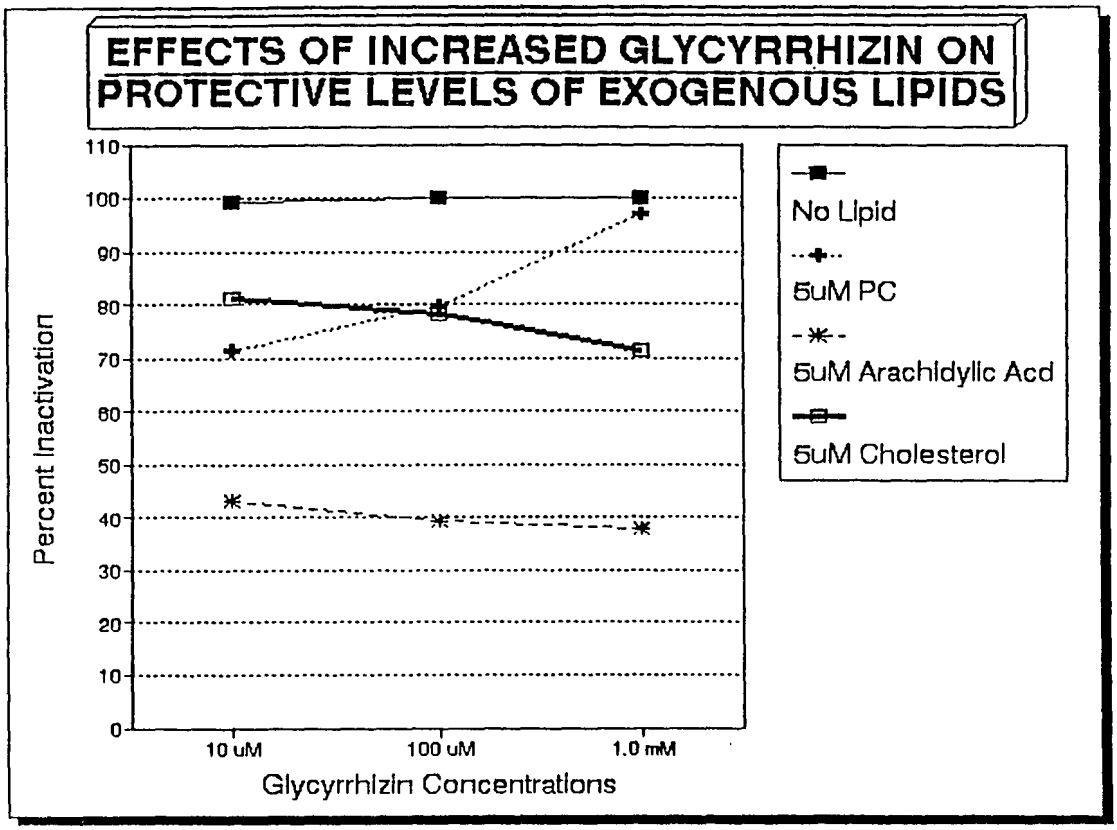
PLAQUES PER PLATE AND PERCENT INACTIVATION

Lipid	PFU/ml Plated [Φ6]	GLYCYRRHIZIN INCUBATION CONCENTRATIONS 0.0μM PLATES 1	2	10μM 1	2	100μM 1	2	1.0mM 1	2
CONTROL									
	$5x10^2$	680	820	8	4	0	1	0	1
	$5x10^1$	62	71	0	1	0	0	0	0
	$5x10^0$	5	9	0	0	0	0	0	0
Average ($nx10^0$)		7.05		0.055		0.002		0.001	
% Inactivation		0.0%		99.25%		100%		100%	
Phosphatidyl Choline	$5x10^2$	640	730	290	220	146	186	31	31
	$5x10^1$	78	69	2	1	1	0	0	0
	$5x10^0$	4	7	0	0	0	0	0	0
Average ($nx10^0$)		6.57		2.03		1.44		0.23	
% Inactivation		6.8%		71.2%		79.6%		96.8%	
Arachidylic Acid									
	$5x10^2$	750	870	570	530	610	400	490	460
	$5x10^1$	55	62	3	2	2	5	3	5
	$5x10^0$	6	8	0	0	0	0	0	0
Average ($nx10^0$)		6.98		4.01		4.27		4.37	
% Inactivation		0.9%		43.1%		39.5%		38.0%	
Cholesterol									
	$5x10^2$	850	890	180	120	170	190	270	240
	$5x10^1$	49	77	1	0	1	0	1	2
	$5x10^0$	6	11	0	0	0	0	0	0
Average ($nx10^0$)		7.83		1.33		1.54		2.03	
% Inactivation		-1.1%		81.2%		78.2%		71.2%	

Control plates contained 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin. The above data was obtained using the plaque assay at an incubation time of 30 minutes; incubation temperature of 20°C; respective incubation concentration of glycyrrhizin of 0μM, 10μM, 100μM and, 1mM; a phage Φ6 incubation concentration of $5x10^5$ PFU/ml; and a 5μM incubation concentration of each respective exogenous lipid, except for a 0.0μM concentration for the control plate. There were three plated concentrations of phage Φ6 namely, $5x10^2$ PFU/ml, $5x10^1$ PFU/ml, and $5x10^0$ PFU/ml with host HB10Y. Two plates at each concentration were made. A count of plaques per plate was made. An average was calculated from plaque count data from all plated concentrations and both plates and normalized to the $nx10^0$ PFU/ml plate count.

d.) Graph Section

GRAPH 6. Summary of the effects of increased concentrations of glycyrrhizin in the presence of a protective level of an exogenous lipid.



Control plates contained 0.0μM of the respective exogenous lipid and 0.0μM of glycyrrhizin. Data for the above graph is found in Table 12 and was obtained using the plaque assay at an incubation time of 30 minutes; incubation temperature of 20°C; respective incubation concentration of glycyrrhizin of 0μM, 10μM, 100μM and, 1mM; a phage Φ6 incubation concentration of $5x10^5$ PFU/ml; and a 5μM incubation concentration of each respective exogenous lipid, except for a 0.0μM concentration for the control plate. There were three plated concentrations of phage Φ6 namely, $5x10^2$ PFU/ml, $5x10^1$ PFU/ml, and $5x10^0$ PFU/ml with host HB10Y. Two plates at each concentration were made. A count of plaques per plate was made. An average was calculated from plaque count data from all plated concentrations and both plates. From calculated averages and control plates a percent inactivation was calculated.

e.) Results Summary

At 10μM concentration, glycyrrhizin alone inactivates Φ6 to practically 100%.

From Graph 6 one can see that over a two order of magnitude increase of glycyrrhizin, both arachidylic acid and cholesterol maintained their protective level against the virucidal activity of glycyrrhizin. In particular, with 10μM concentration of arachidylic acid the percent inactivation of Φ6 by 10μM of glycyrrhizin was reduced from 100% to an average of about 40% (+/- 10%). With a constant 10μM concentration of arachidylic acid and increasing concentrations of glycyrrhizin through 100μM and up to 1mM, the percent inactivation was maintained at an average of 40% (+/- 10%). Also, with 10μM concentration of cholesterol the percent inactivation of Φ6 by 10μM of glycyrrhizin was reduced from 100% to an average of about 75% (+/- 10%). With a constant 10μM concentration of cholesterol and increasing concentrations of glycyrrhizin through 100μM and up to 1mM, the percent inactivation was maintained at an average of 75% (+/- 10%).

Phosphatidyl choline behaved differently. As glycyrrhizin concentrations increased from 10μM to 1.0mM the percent inactivation recovered from 70% back near 98% while the concentration of PC was held constant throughout.

f.) Conclusions and Discussion

One can easily see that the virucidal activity of glycyrrhizin is sensitive and reduced by certain lipids. Some of these lipids, to which glycyrrhizin is sensitive, can maintain their neutralizing ability against a hundred fold increase in glycyrrhizin. This is an unexpected result. I had expected that the lipids would alter virucidal activity and in particular the virucidal activity of glycyrrhizin. I had also anticipated that lipid effects would reduce the virucidal activity of glycyrrhizin. I had also expected that certain lipids would be more effective and others uneffective.

The results that are unexpected are that for some lipids, relatively low concentrations are able to reduce the virucidal activity of glycyrrhizin by 25% to 60%. The concentration at which this occurs is equal to the concentration of glycyrrhizin. It is also unexpected that, although the concentration of the virucidal agent, glycyrrhizin, is increased some lipids maintain their protective or neutralizing ability.

Three speculative models to explain the neutralizing phenomenon were presented in the previous Discussion Section

of this thesis. The speculative model which more closely explains these results is not immediatly obvious . The aggregate model seems most plausible simply based upon the natural occurance of glycyrrhizin found in glycoside compounds. There may be more dynamics involved among phage, glycyrrhizin and the protective lipids.

One must at least first, propose, and second, consider, possibilities even though due consideration may give rise to evidence that the possibility is not probable or even reasonable. In considering a possibility with respect to protective effects of certain lipids, one important thing to remember is that the virucidal activity of glycyrrhizin is being reduced by a percentage and not that glycyrrhizin is rendered completely inactive. At least some molecules are still quite active and do inactivate phage from at least 40% to 75%. What is being measured is virucidal activity in terms of percent inactivation. 'Viral Inactivation' is being measured. Also, viral inactivation is reduced but not eliminated. The dynamics of molecules cannot be directly explained from these inactivation measurements because first the dynamics of interaction of glycyrrhizin with Φ6 surface molecules must be examined. What is the turn over rate or dynamics and also the molecular mechanical dynamics of glycyrrhizin molecules involved in phage inactivation and with neutralizing-lipids?

If protective lipids and glycyrrhizin yield a virucidally inactive change to glycyrrhizin then there are static factors in virucidal neutralization. If protective lipids interact dynamically with glycyrrhizin then there is no inactivation of glycyrrhizin and its potential as a virucidal compound has a dynamic rate. Likewise, if the virucidal activity of glycyrrhizin yields a chemically static change to glycyrrhizin such that after the participation of a single glycyrrhizin molecule with Φ6 surface molecules it becomes virucidally inactive then this is modeled as a static factor. If one glycyrrhizin molecule can repeatedly participate in inactivating, in this case, dislodging phage protein then its potential for inactivating phage is not only concentration dependent but time dependent as well. These parameters are directly dependent upon molecular mechanisms.

The main point being raised, but not concluded, is that incubation time was also held constant. In a dynamic system there are turn over rates and time factors are usually involved. Experiments which examine incubation time as a variable may indicate that the virucidal activity of glycyrrhizin at a given temperature and concentration may be reduced or rather slowed down in the presence of a protective lipid. This means that to recover the same

virucidal inactivation levels before neutralizing lipid is added simply adding more glycyrrhizin may not be sufficient. Possibly, in the presence of certain protective molecules, a longer incubation time might yield the recovery of original inactivation values of a virucidal compound rather than increased concentration.

A comprehensive formula which identifies and characterizes all the variables involved in the virucidal potential or virucidal dynamics of a compound can begin as a function of several simple variables. Virucidal potential is a function of these variables despite their complexities.

In order to begin to identify and characterize the relationship between variables in antiviral research a theoretical mathematical model can be described. A starting point in the identification of such a function can be described by an Inactivation Potential formula (IP).

Inactivation Potential Formula

$$IP = f(IC_v, FC_v, IC_a, FC_a, IC_n, FC_n, IT, It, IC_p, IC_s)$$

IP - inactivation potential is expressed as a percent

IP = 100% would be 0 plaques w/ control plates

For simplicity incubation and final concentrations of neutralizing agents, synergistic agents and potentially unwanted antiviral agents are not introduced in our initial early experiments and It, IT are held constant; therefore IP formula can be simplified as:

$$IP_1 = f_1(IC_v, FC_v, IC_a, FR_a)$$

What is attempted to be examined first is each parameter individually while holding all others constant:

$$IP = f_{1a}(IC_v)$$
$$IP = f_{1b}(FC_v)$$
$$IP = f_{1c}(IC_a)$$
$$IP = f_{1d}(FC_a)$$

After or in determining the effects of this independent variables alone, the effects of the relationships between these can be considered:

$IR_{v/a}$ The ratio of virus concentration to antiviral agent concentration at the time of incubation.

There are relationship between certain parameters that dictate that other parameters in the IP function cannot be overlooked. For instance, if a final plated concentration is equal to an incubation concentration that is showing some effect then the effects of some final plated concentrations diluted from higher incubation concentrations cannot be cannot be ignored.

Tube Number	1	2	3	4
Incubation Concentration	10,000	1000	100	10
Dilution by 100	÷ 100	÷ 100	÷ 100	÷ 100
Final Plated Concentration	100	10	1	0.1
Effect (Yes/No)	YES	YES	YES	NO

Note that there was an effect noted when incubating at 10,000; at 1000; and at 100. But also observe that the Incubation Concentration of 100 which has an effect is also one of the Final Plated Concentrations which observations are based upon. Hence, if an IC_n exist which equals a FC_m then IC_n results cannot be simply accepted. In the above example $IC_3 = FC_1$.

Essentially if there exists an IP > 10% for IPCO(N,x) and IPCO(x,M) exists. That is if there is a nominal percent inactivation from a given incubation concentration and there also exists a final concentration equal to that same

incubation concentration showing nominal percent inactivation.

Also if the effects of a potentially unwanted virucidal compound affect inactivation percentages greater than (+/-) 1% then IC_p cannot be ignored or:

if $IP(IC_p = X) - IP(IC_p = 0) > 1\%$

then $IC_p = X$ cannot be ignored.

and also with respect to ratios:

$FC_{v/a}$ and $FC_{v/p}$ cannot be ignored if ((FC/IC) > 0.01),

that is, if the incubation concentration has not been diluted by a factor of 100 or more.

In addition the formula of the IP function would include various rates such as the rate at which phage is inactivated by a virucidal agent. This rate PIR, phage inactivation rate for example, would be experimentally determined and its units would be PFU/ml/sec.

PIR - phage inactivation rate PFU/ml/second at STP, no IC_n

An example formula might be:

$$IP = \left(PIR \times e^{g(IT,It) \times (ICa/ICv)} \right) - e^{g(IT,It) \times (ICn/ICa)}$$

As an exam[ple the inactivation potential may exponentially increases by the incubation ratios of antiviral agent to virus multiplied by an inactivation rate factor and subtracted (reduced by) by the exponentially increasing incubation concentration ratio of a neutralizing agent to antiviral agent. The exponential increase is multiplied by a factor g(), which is a function of incubation time and temperature.

In summary, variables that effect virucidal activity of a potential virucidal compound must be identified, characterized and measured. From empirical and experimental data a parameterized formula can be constructed to model inactivation potential as a function of these variables.

Experiments in this thesis have shown that parameters of the IP formula do exist and represent variables involved in determining virucidal activity. This research has also shown that some variables act upon specific aspects of virucidal activity and sometimes only under certain conditions. Each variable can be dependent or independent. Each variable can be expressed as a value, a constant, be characterized by a rate or an equation. Most of these variables at this point would need to be determined experimentally. The basis of identifying variables, characterizing virucidal dynamics and formularizing inactivation potential is to move more closely to a predictive model.

Retinoid and PM2 Studies

a.) Rationale and Hypothesis

Previous work in this lab had shown that retinoids were generally effective against lipid containing viruses (1, 4). At that time all-trans isomers of retinal, retinol and retinoic acid were studied. In addition some retinoids were also shown to be effective against protein-coated viruses such as PM2 and SV40. Retinoic acid (all trans) was the most active retinoid against PM2, which is protein-coated and contains an internal lipid bilayer. Retinoids are amphipathic compounds which have a polar and functional R group at the end of the hydrocarbon tail. In order to investigate the effective structural nature of amphipathic compounds both the conformation of the hydrocarbon tail and functional groups between conformations were studied for their virucidal activity against phage PM2.

I had hypothesized that the cis-isomer configurations would be less effective than their all-trans isomer equivalents at intercalating into the viral protein coat due to the isomeric kink in the hydrocarbon tail and therefore would be less effective at inactivating the virus.

I had also hypothesized that the retinoic acid functional group would remain the most effective functional

group, even within the cis-isomer group, at interacting within the protein-coat and causing viral inactivation. This evidence would also serve to further elucidate the potential mechanisms by which the retinoids are interacting with PM2.

b.) Experimental Design and Method

Concentrations of 0.0μM, 0.1μM, 1.0μM, 10.0μM, and 100μM of cis and trans isomers of retinoids were tested to determine their respective percent inactivation curve.

The retinoids studied are retinal, 9-cis-retinal, 13-cis-retinal; all-trans-retinol, 13-cis-retinol; all-trans-retinoic acid and 13-cis-retinoic acid.

The general plaque assay was followed. Incubation tubes containing 5×10^5 PFU/ml of phage PM2 were prepared at a volume of 10ml. The respective concentrations listed above of each retinoid were added at time t=0 and vortexed. All tubes incubated for 30 minutes. Final plating concentrations of 5×10^3 PFU/ml and 5×10^1 PFU/ml of the above tubes were made by dilution. 0.1ml of phage concentration samples were added to 10ml top agar tubes which already contained 0.4ml of bacterial host which was then vortexed and immediatly plated. Top agar tubes were cooled to 45°C before either host or phage were added. Plates sat for 24-36 hours and then plaques were counted.

The concentrations used were all soluble in Q-Medium (see Appendix A) which was used for host cell growth. The 0.0μM concentration of each retinoid were used as a control

and to normalize percent inactivation. This has yielded an inactivation curve for each retinoid on PM2.

In addition experiments were done to test the effects of each retinoid on normal host lawn growth without any phage present. This was done to ensure that plaque counts and host lawn morphology is soley due to phage inactivation and not from interference by any of the retinoids with normal host growth.

c.) Data Section

TABLE 13-A. Retinal (all trans) Plaque Counts.
Plaque assay data counting plaques per plate.

PLAQUES PER PLATE - RETINAL (ALL TRANS)

Retinal all-trans Incubation Concentration	PLATED CONCENTRATION 5×10^1 PFU/ml EXPERIMENTAL RUN 1	5×10^1 PFU/ml EXPERIMENTAL RUN 2	5×10^0 PFU/ml EXPERIMENTAL RUN 1	5×10^0 PFU/ml EXPERIMENTAL RUN 2	PERCENT INACTIVATION
0.0 μM	47	58	5	6	0%
0.1 μM	47	48	5	4	9%
1.0 μM	51	42	4	5	11%
10 μM	53	49	4	5	3%
100 μM	42	44	4	4	19%
1.0 mM	30	37	2	4	36%
10 mM	34	35	3	3	34%

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of 5×10^5 PFU/ml.

There were two plated concentrations of phage PM2 namely, 5×10^1 PFU/ml and 5×10^0 PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

TABLE 13-B. 9-cis Retinal Plaque Counts.
Plaque assay data counting plaques per plate.

PLAQUES PER PLATE - 9-cis RETINAL					
9-cis Retinal Incubation Concentration	PLATED CONCENTRATION 5×10^1 PFU/ml		5×10^0 PFU/ml		PERCENT INACTIVATION
	EXPERIMENTAL RUN 1	2	1	2	
0.0 μM	55	58	6	6	0%
0.1 μM	55	46	5	5	10%
1.0 μM	60	41	5	5	10%
10 μM	62	49	4	6	2%
100 μM	53	46	4	4	6%
1.0 mM	42	39	3	4	28%
10 mM	46	38	3	4	25%

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of 5×10^5 PFU/ml.

There were two plated concentrations of phage PM2 namely, 5×10^1 PFU/ml and 5×10^0 PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

TABLE 13-C. 13-cis Retinal Plaque Counts.
Plaque assay data counting plaques per plate.

PLAQUES PER PLATE - 13-cis RETINAL					
13-cis Retinal Incubation Concentration	PLATED CONCENTRATION 5×10^1 PFU/ml		5×10^0 PFU/ml		PERCENT INACTIVATION
	EXPERIMENTAL RUN 1	2	1	2	
0.0 μM	64	66	7	6	0%
0.1 μM	65	56	6	5	7%
1.0 μM	68	51	6	5	8%
10 μM	67	53	6	6	8%
100 μM	63	56	6	5	9%
1.0 mM	55	49	5	4	20%
10 mM	56	51	6	4	18%

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of 5×10^5 PFU/ml.

There were two plated concentrations of phage PM2 namely, 5×10^1 PFU/ml and 5×10^0 PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

TABLE 13-D. Retinol (all-trans) Plaque Counts.
Plaque assay data counting plaques per plate.

PLAQUES PER PLATE - RETINOL (ALL-TRANS)					
Retinol all-trans Incubation Concentration	PLATED CONCENTRATION 5×10^1 PFU/ml		5×10^0 PFU/ml		PERCENT INACTIVATION
	EXPERIMENTAL RUN 1	2	1	2	
0.0 μM	68	79	8	8	0%
0.1 μM	69	70	7	7	5%
1.0 μM	74	65	7	7	6%
10 μM	67	73	6	7	5%
100 μM	68	69	5	5	7%
1.0 mM	39	32	4	4	52%
10 mM	24	BP	2	1	65%

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of 5×10^5 PFU/ml.

There were two plated concentrations of phage PM2 namely, 5×10^1 PFU/ml and 5×10^0 PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

TABLE 13-E. 13-cis Retinol Plaque Counts.
Plaque assay data counting plaques per plate.

PLAQUES PER PLATE - 13-cis RETINOL					
13-cis Retinol Incubation Concentration	PLATED CONCENTRATION 5×10^1 PFU/ml		5×10^0 PFU/ml		PERCENT INACTIVATION
	EXPERIMENTAL RUN 1	2	1	2	
0.0 μM	57	66	6	8	0%
0.1 μM	58	67	6	7	0%
1.0 μM	63	63	7	7	0%
10 μM	55	58	6	7	8%
100 μM	49	51	5	5	19%
1.0 mM	33	32	3	4	47%
10 mM	25	21	2	1	56%

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of 5×10^5 PFU/ml.

There were two plated concentrations of phage PM2 namely, 5×10^1 PFU/ml and 5×10^0 PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

TABLE 13-F. Retinoic Acid (all-trans) Plaque Counts. Plaque assay data counting plaques per plate.

PLAQUES PER PLATE - RETINOIC ACID (ALL-TRANS)					
Retinoic Acid all-trans Incubation Concentration	PLATED CONCENTRATION 5×10^3 PFU/ml		5×10^1 PFU/ml		PERCENT INACTIVATION
	EXPERIMENTAL RUN 1	2	1	2	
0.0 μM	>>>	>>>	55	61	0%
0.1 μM	>>>	>>>	25	31	51%
1.0 μM	>>>	>>>	14	17	73%
10 μM	>>	@180	2	1	97%
100 μM	13	18	0	0	99%
1.0 mM	0	1	0	0	100%
10 mM	BP	BP	BP	BP	---

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of 5×10^5 PFU/ml.

There were two plated concentrations of phage PM2 namely, 5×10^3 PFU/ml and 5×10^1 PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

TABLE 13-G. 13-cis Retinoic Acid Plaque Counts.
Plaque assay data counting plaques per plate.

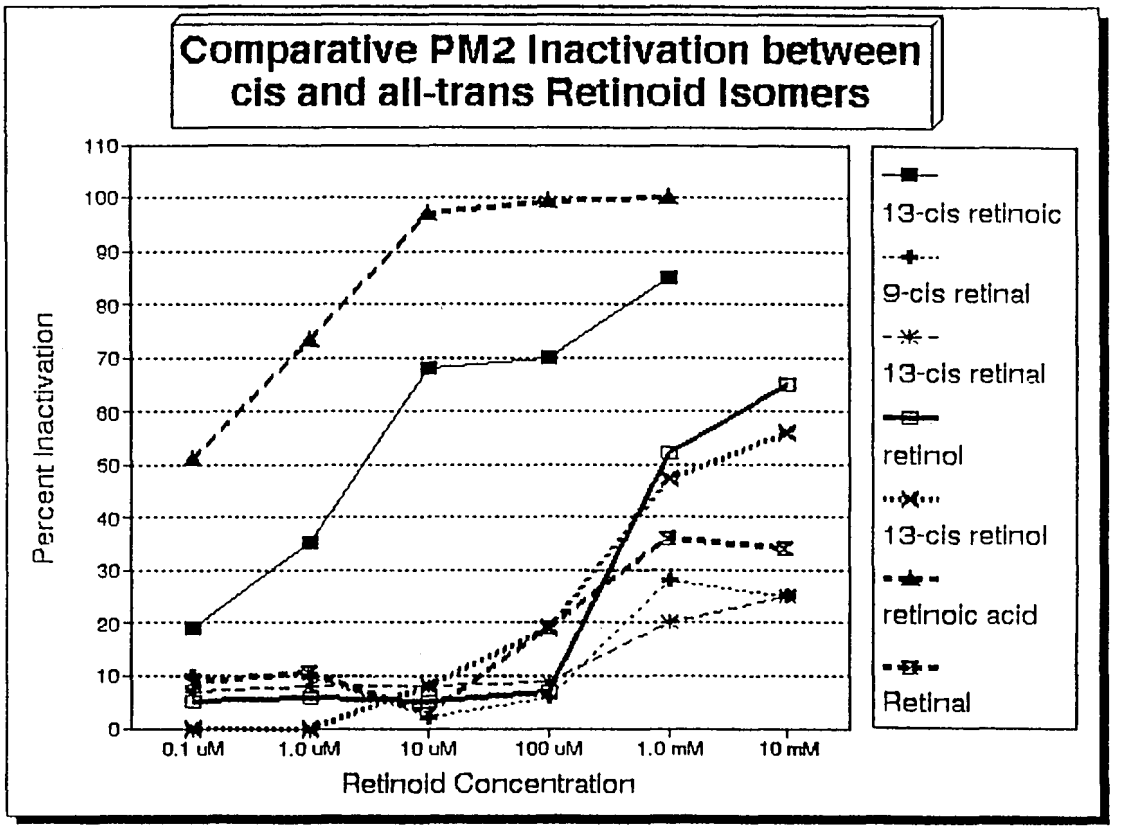
PLAQUES PER PLATE - 13-cis RETINOIC ACID					
13-cis Retinoic Acid Incubation Concentration	PLATED CONCENTRATION 5×10^1 PFU/ml		5×10^0 PFU/ml		PERCENT INACTIVATION
	EXPERIMENTAL RUN 1	2	1	2	
0.0 μM	50	55	5	6	0%
0.1 μM	39	46	5	5	19%
1.0 μM	31	37	4	4	35%
10 μM	13	20	2	2	68%
100 μM	13	18	2	2	70%
1.0 mM	6	10	1	1	85%
10 mM	10	BP	BP	BP	---

The above data was obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of 5×10^5 PFU/ml.

There were two plated concentrations of phage PM2 namely, 5×10^1 PFU/ml and 5×10^0 PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

d.) Graph Section

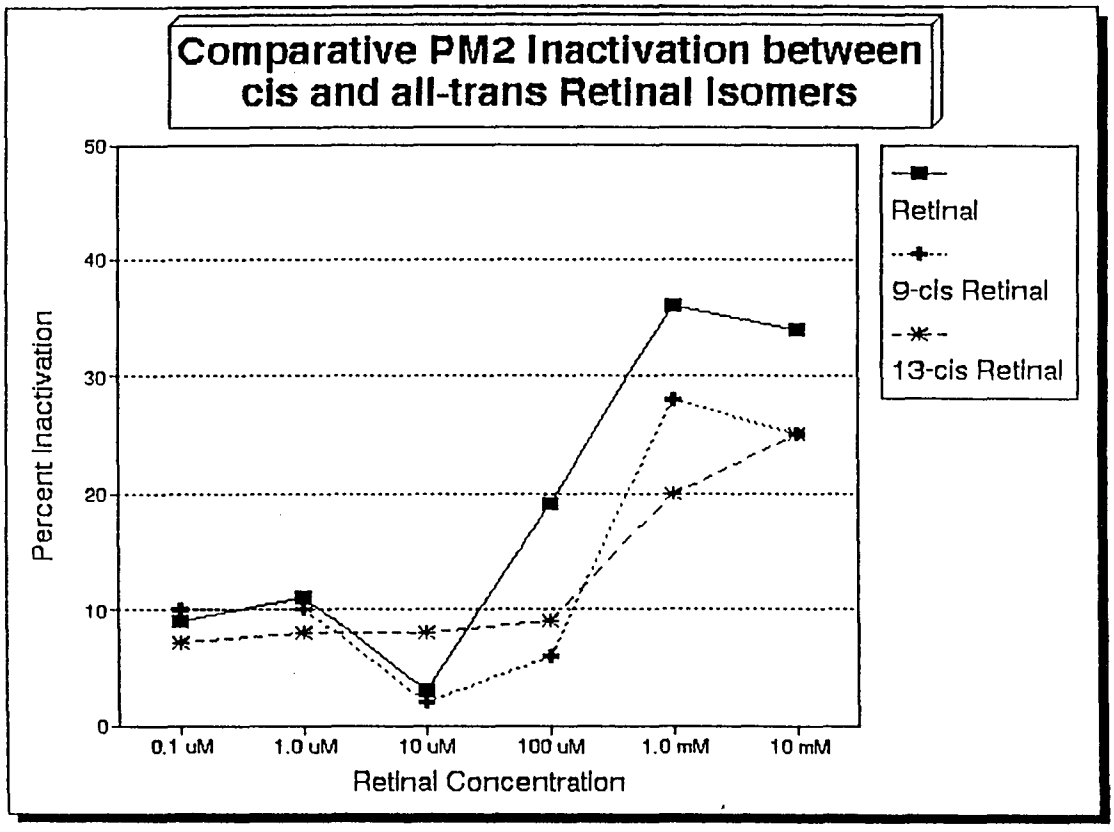
GRAPH 7-A. Percent Inactivation Curve of all Retinoids Tested upon phage PM2.



Data for the above graphs were obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of $5x10^5$ PFU/ml.

There were two plated concentrations of phage PM2 namely, $5x10^1$ PFU/ml and $5x10^0$ PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

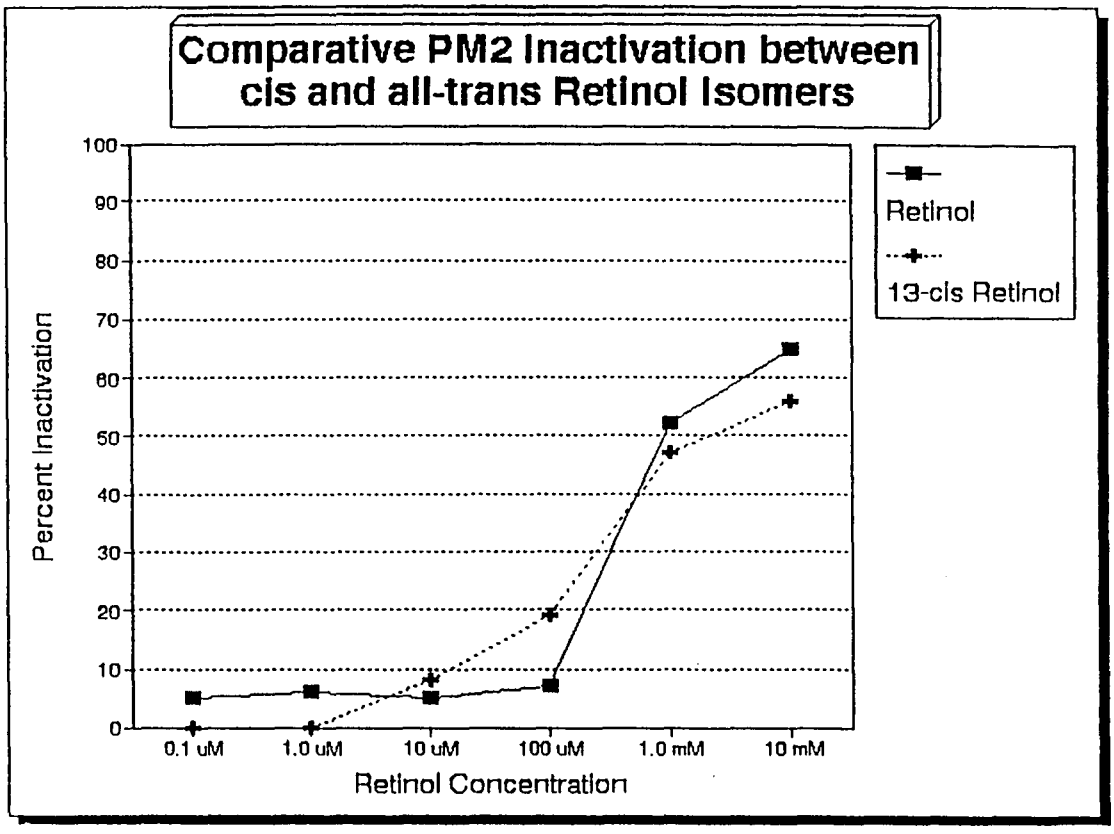
GRAPH 7-B. Percent Inactivation Curve of all Retinal Isomers Tested upon phage PM2.



Data for the above graphs were obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of $5x10^5$ PFU/ml.

There were two plated concentrations of phage PM2 namely, $5x10^1$ PFU/ml and $5x10^0$ PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

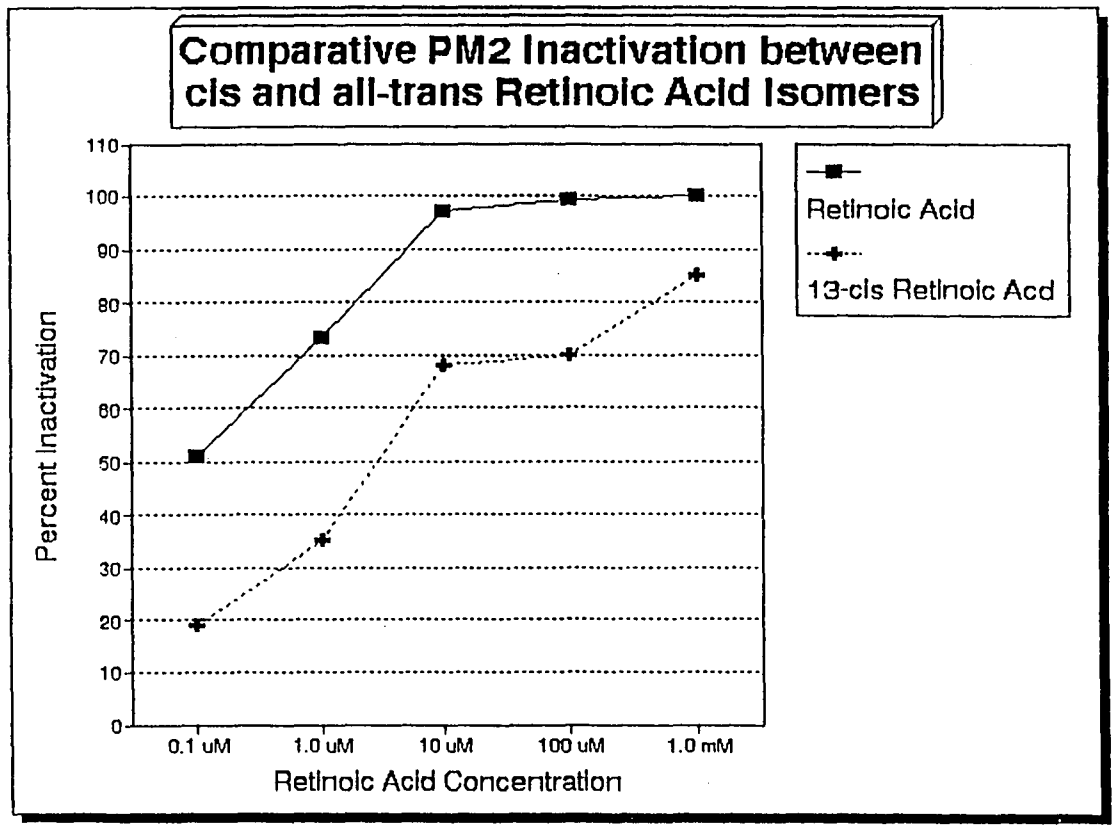
GRAPH 7-C. Percent Inactivation Curve of all Retinol Isomers Tested upon phage PM2.



Data for the above graphs were obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of $5x10^5$ PFU/ml.

There were two plated concentrations of phage PM2 namely, $5x10^1$ PFU/ml and $5x10^0$ PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

GRAPH 7-D. Percent Inactivation Curve of all Retinoic Acid Isomers Tested upon phage PM2.



Data for the above graphs were obtained using the plaque assay at an incubation time of 30 minutes, incubation temperature of 20°C, and a phage PM2 incubation concentration of $5x10^5$ PFU/ml.

There were two plated concentrations of phage PM2 namely, $5x10^1$ PFU/ml and $5x10^0$ PFU/ml with host BAL31. The experiment was run two times at seven concentrations of the respective retinoid. A count of plaques per plate were made. An average of percent inactivation was calculated from an average of plaque count data from each plated concentration, and both runs, and a percentage was then based on 0.0μM control at 0% inactivation.

e.) Results Summary

Retinoic acid (all-trans) is the most active virucidal agent of the retinoids tested against phage PM2. The 13-cis isomer of retinoic acid is the second most virucidal agent and is more active than all other isomers of retinal and retinol.

Table 14. Comparison of Percent Inactivation among all the Retinoids Tested upon phage PM2.

RETINOID PERCENT INACTIVATION COMPARISON	
Retinoid Isomer	Percent Inactivation
Retinoic Acid (all-trans)	99%
13-cis Retinoic Acid	70%
Retinal (all-trans)	19%
13-cis Retinol	19%
13-cis Retinal	9%
Retinol (all-trans)	7%
9-cis Retinal	6%

Data for this table is taken from the 100μM concentration of each retinoid isomer tested in Tables 13-A to 13-G. Table 14 is listed in descending order of percent inactivation.

By examining Graph 7-B, 7-C and 7-D one can see that overall the all-trans isomer of each retinoid demostrated greater virucidal activity than its respective cis-isomers.

A general ranking among the three retinoid groups tested is (1st) Retinoic Acid, (2nd) Retinol, and (3rd) Retinal. Slight variations in ranking at certain data

points within the inactivation curves may exist but overall averages yield the above general ranking.

f.) Conclusion and Discussion

Previous work in this lab has shown that retinoids, especially retinoic acid, showed a substantial virucidal activity against the phage PM2. These retinoids have also been shown to be effective against other protein coated viruses such as SV40 (1, 4). In this section of this thesis research the virucidal activity of the cis-isomer configurations were studied and compared to their corresponding all-trans isomer results. Retinoids are amphipathic compounds which have a functional R group at the end of its polar tail. Comparing results of virucidal activity between retinoid functional groups as well as changes in isomeric conformations indicate that the acid group COOH and the all-trans isomers are the more effective components of the retinoid compounds tested against the protein-coated phage PM2. This suggests that these components may be, in general, more effective against other protein coated viruses as well.

It was hypothesized that the cis-isomer configurations would be less effective than their all-trans isomer equivalents. This is not the case for the retinal and retinol isomers. The cis-isomers of retinal and retinol did not show a greater nor a substantially less effective virucidal activity compared to their all-trans isomers. The

13-cis retinoic acid did however, show an appreciable reduction in percent inactivation than the all-trans isomer of retinoic acid. The explaination for this reduced activity is most likely due to the decreased ability of the 13-cis isomer of retinoic acid at intercalating into the viral-protein-coat due to the isomeric kink in the hydrocarbon tail. The cis-isomer kink may cause a resistance or require additional energies in order to intercalate into the protein coat. In addition the cis-isomeric kink may distort the ability of the functional polar group to interact with either the protein outer coat or, after intercalation, the interaction with the inner lipid membrane. As previously stated other researchers believe that the retinoids are interacting with protein, specifically the protein composing the outer-coat of the virion.

I had also hypothesized and shown, in this research, that the retinoic acid functional group is the most effective functional group. This research supports previous work by showing that all-trans retinoic acid was the most active retinoid against the protein-coated phage PM2. Other researchers and myself speculate that the interaction of the retinoid polar tails within the protein-coat lead to viral inactivation. A proposed mechanism by which the retinoids are interacting with phage PM2 and lead to virion disruption

may begin with intercalation into the protein coat then followed by possible penetration and interaction with the internal lipid bilayer of the phage PM2.

Retinoic acid isomers were the most active against phage PM2 but the least active against phage Φ6. Retinol and retinal where the least active against phage PM2 but the most active against phage Φ6. The outer structure of phage PM2 is protein, whereas the outer structure of phage Φ6 is lipid. Glycyrrhizin was more effective against phage Φ6 than phage PM2. Both phage Φ6 and herpes virus possess external lipid-enveloped structures and were comparatively more susceptible to both retinol and glycyrrhizin. Phage PM2, with its exterior protein coat, was comparatively less susceptible to both retinol and glycyrrhizin. The COOH R-group of retinoic acid is the most effective retinoid tested upon the protein-coated phage PM2 and, although untested, speculatively the cis-isomer would also be effective upon SV40 virus as well. I propose as a possible explanation that the protein of the outer protein-coat may require the activity and exposure of the COOH R group in order to be more affected by polar groups of retinoids and cause protein coat disruption.

Although glycyrrhizin was less effective on PM2 than Φ6, it did show virucidal activity. The amphipathic nature

of glycyrrhizin does include a COOH group, as well as the -OH group. This supports the speculative conclusion concerning the effectiveness of the acid COOH group on PM2. The molecular availableness or exposure of the COOH group most likely has some contribution to the effectiveness of glycyrrhizin. This can be further substatiated since the cis-isomer of retinoic acid shows a reduced activity (reduced exposure due to kink) and that glycyrrhizin shows a respectable activity against PM2 due to the COOH group although less susceptability than Φ6 due to a greater sensitivity of Φ6 to the -OH group.

The hypothesis of this research has been investigated and verified and has provided some evidence to further suggest the nature of retinoid inactivation mechanisms upon phage PM2. In addition, both objectives of (1.) determining how the virucidal activity of the cis-isomers of the retinoids compare with all-trans isomer results on phage PM2 and (2.) which functional group shows greater and consistent virucidal activity upon the protein-coated phage PM2, have been completed.

APPENDIX A

MEDIUM PREPERATION

Q Medium is used for Bal31,
Alteromonas Espejiana, Host forPM2 phage
V Medium is used for HB10Y,
Phaseolicola, Host for Φ 6 phage

Liquid Medium Ingredients per 1 Liter distilled water.

Component	Q Medium	V Medium
Tryptone	10g	10g
Yeast Extract	5g	5g
NaCl	26g	7g
KCl	0.7g	1.5g
MgSO	12g	0.5g
CaCl	1.5g	0

Bottom Agar for Plates.
Use above Liquid Medium ingredients plus 2.0% Agar.
Agar 20g 20g

Top Agar
Use above Liquid Medium ingredients plus 1/2% Agar.
Agar 5g 5g

APPENDIX B

GENERAL VIRUCIDAL TEST PROCEDURE EFFECT OF VARIOUS CONCENTRATIONS OF A COMPOUND ON A PHAGE-HOST SYSTEM

Materials:

- 20 sterile tubes
- 4 bottom agar plates
- 4 top agar tubes (7-10ml) each
- liquid medium
- retinoid componds
- 8 sterile 1ml pipets

Protocol:

Preparation Step

* Put Top Agar in Large Beaker of Boiling water and thoroughly melt Top Agar Medium. (PS1.01)

Four sterile tubes should be placed in temperature bath and allowed to be pre-heated. (PS1.03)

After step (S6.0X) dispense 7-10ml into each of 4 sterile tubes and place in temperature bath at (50 C). (PS2.02)

* Host Bacteria Preparation (PS3.00)

a. Take 1ml of stock bacteria and add to 9ml of medium into a sterile capped flask.
b. Flame flask lip and recap.
c. Put incubation flask on shaker for 1-2 hrs.

It may require up to 10 - 18 hrs depending upon how long bacterial stock has been in refrige.

Double above 1-2hr time for each week in refrige.

1. Take 0.1ml of phage stock and then add into 10 ml of medium in a tube. Vortex. When ever vortexing pinch finger and thumb above liquid level and below sterile cap to create a vortex node so liquid will not spin above pinched point. Return Stock to refrigerator. (S1.01)

2. Take 0.1ml of above dilution (S1.01) and then add into 40ml of medium into a tube. Vortex. (S2.01)

3. Dispense 10ml of (S2.01) dilution into each of 4 sterile tubes. (S3.01)

The PFU/ml in each of the 4 tubes is about
1.2E10 / (100x * 400x) = 3 x 10^5 PFU/ml

4. Label the 4 tubes from (S3.01) with each respective concentration, for example if using ethenol:
0%, 0.4%, 1.0%, 0.4% (S4.01)

5. At a time t=0 we will add different concentrations of the compound to each of the 4 tubes and let them sit for t=15 minutes at which time we will dilute the 4 tubes containing phage and plate.

6. Using the ethenol concentrations as an example:
At t=0 add 0.00ml of compound to the 0.0% tube (S6.01)
0.04ml of compound to the 0.4% tube (S6.02)
0.10ml of compound to the 1.0% tube (S6.03)
0.40ml of compound to the 4.0% tube (S6.04)

At this point the tubes contain medium, 3 x 10^5 PFU/ml phage, and various (for example 0% to 4%) concentrations of the compound.

7. At t=15 minutes dilute 0.1ml from each of 4 (S6.0X) tube into 4 respective tubes containing 10ml medium. (S7.01)
This is another dilution of 100. (10 / 0.1)

The PFU/ml of these 4 new tubes is 3 x 10^3 PFU/ml.

8. Retrieve the 4 top agar tube from the temperature bath that were prepared in step (PS1.02).
Add 0.4ml of host bacteria to each of the four top agar tubes.
Add to the (in)side of the tube and allow to run down into the agar.
Do not Vortex. (S8.01)

9. Have pipets already sitting in each of the phage dilution tubes. Take 0.1ml from the (S7.01) tubes and pipet down into the 7-10ml top agar tubes for each (S7.01) tube. Immediately Vortex for 2 sec. seconds and Quickly Plate. (S9.01)

This is another dilution of 100. (10 / 0.1)
The final concentration is 3 x 10^1 PFU/ml. This means about 30 plaques for the 0.0% compound control.

10. Incubate plaques overnight. (S10.01)

11. Calculate percent survival for each compound concentrations as follows:

$$\% \text{ survival} = \frac{\text{surviving PFU/ml in [compound]}}{\text{contol 0.0\% PFU/ml}}$$

APPENDIX C

VIRUS ABBREVIATIONS

HSV-1	herpes simplex 1 virus
VZV	varicella-zoster virus
NDV	Newcastle disease virus
HIV	human immunodeficiancy virus
HBV	hepatitis B virus

APPENDIX D

STANDARD ANTIVIRAL RESEARCH RELATED ABBREVIATIONS

AV	antiviral
AVA	antiviral activity
AVC	antiviral compound
AVF	antiviral factor
pfu	plaque forming units
hpi	hours post infection
IC_{50}	infectious concentration 50%
ID_{50}	infectious dose 50%
LD_{50}	lethal dose 50%
LD_{99}	lethal dose 99%
MIC	minimum inhibitory concentration
PRP	pathogenesis related protein
TCD_{50}	tissue culture dose 50%
virion	virus particle

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Vita

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